Classic galactosemia

Classic galactosemia:

A zebrafish model and new clinical insights

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Contents

Chapter 1

General introduction

CLASSIC GALACTOSEMIA

Though the first description of classic galactosemia dates back to 1908, patient care continues to present challenges that emphasize gaps in our knowledge on the pathophysiological and clinical basis of this rare disease. Classic galactosemia is an autosomal recessive disorder, affecting 1:16.000-60.000 newborns worldwide, and is caused by a profound deficiency of galactose-1-phosphate uridylyltransferase (GALT) (1, 2). Together with galactokinase (GALK) and UDP-galactose 4'-epimerase (GALE) the GALT enzyme constitutes the main route for galactose metabolism, the Leloir pathway. The net result of galactose breakdown is the formation of glucose-1-phosphate (Glc-1-P), which can be further metabolized to produce energy, and UDP-galactose (UDP-gal), which is involved in glycosylation processes.

The severe GALT deficiency in patients with classic galactosemia derives from mutations in the *GALT* gene. Thus far over 300 *GALT* variants have been reported, of which the most prevalent lead to protein misfolding with subsequent increased propensity for aggregation and degradation (3, 4).

Affected newborns develop an acute and potentially lethal syndrome upon exposure to galactose in milk, including failure to thrive, feeding difficulties, liver failure, encephalopathy, and regularly E. coli sepsis. The present standard of care consists of lifelong dietary galactose restriction, which, notwithstanding its irrefutable life-saving role in the acute neonatal toxicity, fails to prevent severe fertility (5-9), brain (10-19) and bone health impairments (20) later in life. These complications represent a heavy burden for patients and their families (21), as well as for health care and society in general.

In order to improve patient outcome, the development of new therapeutic agents is pursued (4, 22, 23). Increased knowledge on the pathogenesis of the disease, as well as insight in the developmental stage at which lesions occur, are crucial for accurate therapeutic strategy establishment. Toxic accumulation of galactose and its metabolites (24, 25), aberrant glycosylation (26, 27), endoplasmic reticulum stress (28, 29), GALT misfolding (3, 4) and dysregulation of gene pathways (30), such as down-regulation of the PI3K-Akt growth signaling pathway (31), have been implicated as potential pathogenic mechanisms. Whether organ damage is established in utero or in early postnatal life, or whether the clinical impairments are the result of ongoing toxicity throughout development, remains to be elucidated.

The two disease models currently available for classic galactosemia, the mouse model (29) and the *Drosophila melanogaster* model (32), have both shown their importance for pathogenesis studies. However, a disease model amenable for organ studies from embryo stage to adulthood and high throughput screening of pharmacological compounds is currently lacking, which hampers progress of galactosemia research.

Furthermore, it has been shown that large discrepancies between countries exist regarding diagnosis, treatment, follow-up and counseling of patients (33). Evaluation of the

extent and implications of the long-term complications and elaboration of clinical recommendations are essential for creating consensual and improved patient care worldwide.

OVARIES

With its prevalence of over 90%, primary ovarian insufficiency (POI) represents the most frequent long-term impairment of classic galactosemia (34). Female patients are already at an early age confronted with the struggles that reduced fertility presents, and it is thus not surprising that POI is considered a major issue by both patients and their families (21).

Though POI is commonly defined as the presence of amenorrhea for at least 4 months in a woman less than 40 years old, accompanied by two serum FSH levels in the menopausal range, it rather reflects a continuum of impaired ovarian function, which is varying, unpredictable and does not exclude pregnancy (35). In general, POI may be due to establishment of a compromised initial pool of primordial follicles in utero or an accelerated follicle apoptosis or destruction after birth (36).

In classic galactosemia there seems to be a rapid decline of ovarian reserve due to a reduction in the number of primordial follicles (6, 8). Pathogenic processes proposed to be involved include impaired primordial germ cell (PGC) development (37), direct toxicity of galactose and its metabolites to oocytes and follicles (38-46), aberrant glycosylation of gene signaling pathways, hormones and receptors involved in ovarian function (26, 27, 30, 47), and dysregulation of the PI3K-Akt growth signaling pathway (31). Elucidating the developmental stage at which ovarian damage occurs, either entirely pre- or postnatal, or an ongoing process throughout development, is essential for counseling and estimating the success rates of potential future interventions. The *Drosophila* melanogaster and mouse models for classic galactosemia do not allow embryonic studies, and therefore novel animal models are needed to provide an answer to this crucial question.

Though POI inherently brings along reduced fertility, pregnancies occur in women despite POI diagnosis with a reported prevalence of 5-10% (48). Also in women with POI due to galactosemia spontaneous pregnancies have been reported multiple times (2, 5, 7, 19, 49-66). Thus far only one small study addressed pregnancy chance in this population and found that most women, despite an active child wish, had not tried to conceive because they had been told this was impossible (7). As much as 44% of the few women who attempted pregnancy was successful, suggesting pregnancy chance might be higher in this population than in women with POI due to other causes.

Gaining more knowledge on pregnancy chance despite POI in this disorder is needed to improve counseling and assess the value of fertility preservation techniques, such as cryopreservation of embryos, oocytes and ovarian tissue, and oocyte donation, in this specific population.

BRAIN

The brain represents the other main target of damage in classic galactosemia. Early case reports demonstrated that severe brain damage occurs in patients when galactose-restriction is not initiated timely. Extensive degeneration of neurons, white matter and cerebellar structures, resulting in microcephaly, mental retardation and gross neurological and motor abnormalities, were reported in two boys with galactosemia and prolonged galactose exposure (67, 68). However, despite early initiation of the diet, nearly all patients encounter one or more of a diverse field of brain impairments. Complications include lower intelligence levels (55, 66, 69-71), speech- and language problems (11-13, 15, 16, 72), slower information processing and memory deficits (69, 73), social and psychiatric difficulties (10, 19, 74), and neurological sequelae (14). Reduced participation in society and impaired ability to build up strong partnerships are generally the result (55), though considerable variability in cognitive functioning exists (69).

Previous imaging studies already demonstrated diffuse atrophy of the cerebrum and ventricle enlargement, cerebellar atrophy, abnormal white matter signal intensity throughout the cerebrum and cerebellum, and focal white matter lesions in a significant part of galactosemia patients (14, 17, 18, 67, 68, 70, 75-79). The pathogenic basis of these abnormalities remains puzzling, however. Recent studies point towards defective white matter microstructure (18), which might result from deficient galactocerebroside formation due to aberrant glycosylation (68, 79), as well as disturbed gray matter density (17). In addition, a functional magnetic resonance imaging study demonstrated significant changes in the language network in patients as compared to healthy controls (16). The broad spectrum of cognitive and neurological problems encountered in this disorder warrants further exploration of functional brain networks that are involved in executive functions, social behavior and psychiatric disorders. Also, fundamental research addressing brain development, myelin composition and neuronal functioning in the galactosemia brain are needed. An adequate animal model that allows organ studies throughout development would contribute to elucidating the fundamental question regarding the timing of damage.

Improved knowledge on the brain networks affected by the disease, as well as on the molecular pathways involved in pathogenesis, would contribute to a better understanding of the neurocognitive profile encountered by patients and create opportunities for the creation of accurate therapeutic or supportive interventions.

BONE

An association between classic galactosemia and decreased bone mass was postulated first in 1993, but until now the extent and clinical relevance for patient care have been undefined.

A bone mineral density (BMD) Z-score of more than 2 standard deviations (SD) below the mean was reported in 25-30% of adult patients (19, 80). Additionally, several studies in children with classic galactosemia support the hypothesis of mildly decreased bone mass from an early age on in these patients (20, 81, 82). Yet, extensive research evaluating the frequency and severity of impaired bone health in patients, as compared to the non-galactosemia population, is lacking.

Acquiring an optimal peak bone mass by maintaining adequate bone health during childhood and adolescence is essential for preventing osteoporosis later in life (83). Patients with classic galactosemia could be at risk for reduced bone mass because of nutritional deficiencies that come along with their diet, POI in females, limited physical activity in some, and possibly intrinsic bone defects associated with the disease (2, 84). The latter is supported by the persistence of reduced bone mass despite adequate supplementation of estrogen and nutrients (84-86). Fundamental studies addressing this potential disease-specific bone defect are, however, very limited. Results from a study in patients with congenital disorders of glycosylation, which demonstrated defective glycosylation of insulin-like growth factor binding protein-3 (87), could have important implications for galactosemia as well, since this disorder secondarily results in glycosylation abnormalities. Future *in vitro* and *in vivo* studies focusing on the pathogenesis of reduced bone mass in classic galactosemia are needed to improve our understanding and management of this complication.

If larger studies indeed confirm an increased propensity for reduced bone mass, careful monitoring and optimization of bone mass seems necessary in these patients from an early age on to prevent the development of osteoporosis and fractures later in life. Recommendations for bone health improvement in this specific population were proposed by our research group in 2007 (88). However, as a result of evolved knowledge on bone mass in classic galactosemia and bone health management in general, these guides warrant revision.

AIMS AND OUTLINE OF THE THESIS

This thesis aims to provide novel perspectives on the long-term complications of classic galactosemia using a combined strategy that encompasses both basic and clinical science. We aim to develop an animal model that is the first to allow both innovative pathogenic studies and pharmacological compound testing. Additionally, we aim to further investigate the extent and clinical implications of the damage to ovaries, brain and bones, thereby providing recommendations for improvement of patient care.

Chapter 2 describes the development of a *galt* knock-out zebrafish model for classic galactosemia. This model allows gaining novel insights in the pathogenesis of this disease, with special focus on the evolvement of organ damage throughout development,

and forms a valuable tool for therapeutic strategy testing. Here, we provide the first new perspectives on the pathogenesis of the long-term impairments of classic galactosemia.

The next two chapters focus on POI and its clinical implications. In chapter 3 the results of an international, epidemiological study on pregnancy chance in women with classic galactosemia and POI are presented. Chance of spontaneous conception was estimated using semi-standardized interviews. Chapter 4 encompasses recommendations regarding the application of fertility preservation techniques in women with classic galactosemia, created with the support of a multidisciplinary team of experts in reproductive medicine.

In chapter 5 we explore functional brain networks of adolescents with classic galactosemia at resting-state using functional magnetic resonance imaging in order to increase our understanding of the puzzling cognitive and neurological impairments seen in these patients.

The last target organ of damage in this disease, the skeletal system, is subject of the next chapters. The results of a systematic review and meta-analysis on bone health in patients with classic galactosemia are presented in chapter 6. The reduced bone mass that is generally present in this population warrants adequate bone health monitoring according to evidence-based strategies, which are proposed in chapter 7.

Lastly, in chapter 8 the most important results of the studies outlined in this thesis are discussed. Furthermore, directions for future research are provided.

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Chapter 2

Impaired fertility and motor function in a zebrafish model for classic galactosemia

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Submitted

ABSTRACT

Classic galactosemia is a genetic disorder of galactose metabolism, caused by severe deficiency of galactose-1-phosphate uridylyltransferase (GALT) enzyme activity due to mutations of the *GALT* gene. Its pathogenesis is still not fully elucidated, and a therapy that prevents chronic impairments is lacking. In order to move research forward, there is a high need for a novel animal model, which allows organ studies throughout development and high-throughput screening of pharmacologic compounds. Here, we describe the generation of a *galt* knockout zebrafish model and present its phenotypical characterization. Using a TALEN approach, a *galt* knockout line was successfully created. Accordingly, biochemical assays confirm essentially undetectable galt enzyme activity in homozygotes. Analogous to humans, *galt* knockout fish accumulate elevated concentrations of galactose-1-phosphate upon exposure to exogenous galactose. Furthermore, without prior exposure to exogenous galactose, they exhibit reduced motor activity and impaired fertility (lower egg quantity per mating, higher number of unsuccessful crossings), resembling the human phenotype of neurological sequelae and subfertility. In conclusion, our *galt* knockout zebrafish model for classic galactosemia mimics the human phenotype at biochemical and clinical levels. Future studies in our model will contribute to improved understanding and management of this enigmatic disorder.

INTRODUCTION

Though its first description dates back to 1908 (1), the pathogenesis of classic galactosemia is still not fully elucidated, and a therapy that prevents chronic impairments is currently lacking. Classic galactosemia is a genetic disorder of galactose metabolism, in which mutations in the *GALT* gene cause a severe impairment of galactose-1-phosphate uridylyltransferase enzyme activity (GALT; EC 2.7.7.12). Patients present during the neonatal period with a toxic syndrome, after ingestion of galactose-containing milk. Though a galactose-restricted diet is life-saving during this phase, it fails to prevent the development of chronic impairments. Target organs of damage are brain, gonads and, to a lesser extent bones, leading to cognitive impairments (2-4), speech and language problems (5-8), neurological sequelae (9), psychosocial difficulties (10), primary ovarian insufficiency in females (11), and mildly decreased bone mass (12). To date, the pathogenic molecular pathways of these complications, as well as the developmental stage at which lesions occur, remain to be elucidated. Toxic accumulation of galactose metabolites, such as galactose-1-phosphate (Gal-1-P) and galactitol (13, 14), aberrant glycosylation (15, 16), endoplasmic reticulum (ER) stress (17, 18), GALT misfolding (19, 20) and dysregulation of the PI3K-Akt growth signaling pathway (21) have been implicated. Improved knowledge is crucial for the establishment of therapies that can prevent damaging effects on the long term. Currently new therapeutic strategies are being explored, including pharmacological/chemical chaperones and GALK inhibitors (20, 22, 23).

Two disease models for classic galactosemia, a fruit fly model (24) and a mouse model (18) have been developed, and both have shown their value (17, 21, 25-28). We have created a zebrafish model for classic galactosemia that complements these existing models. The zebrafish model is an ideal intermediate between the invertebrate (high throughput, evolutionary more distantly related) and the mammalian model system (low throughput, evolutionary closely related) since it is, as a vertebrate, evolutionary more closely related to humans while being a more high-throughput system (high amounts of offspring). Additionally, embryonic development is external and different organ systems are easy to study (transparency and amiability to genetic modification) using reporter lines.

Here, we describe the development of a *galt* knockout zebrafish model and present its phenotypical characterization. This model is meant to improve knowledge on the pathophysiology of chronic impairments, in particular throughout development, and to be used for therapeutic strategy testing.

METHODS

Ethics statement

This study was approved by the Animal Ethics Committee of the University of Maastricht (*Dier Experimenten Commissie*, Maastricht University, Maastricht, The Netherlands) and the Dutch national Central Authority for Scientific Procedures on Animals (*Centrale Commissie Dierproeven*) (AVD107002016545). In addition, approval was obtained from the Animal Ethics Committee of the University of Liège (*Commission d'Ethique de l'Utilisation des Animaux*, Université de Liège, Liège, Belgium; dossier 1576) for the experiments we performed initially in the GIGA Zebrafish Facility and Transgenics, Liège. At all times, the care and use of animals was in agreement with the national and local guidelines. Care of animals and animal experiments were conducted exclusively by licensed technicians and researchers.

Husbandry

Zebrafish were housed in recirculating systems (Fleuren & Nooijen) on a 14/10 day-night regime. Husbandry was essentially performed as described in Lawrence *et al.* (35).

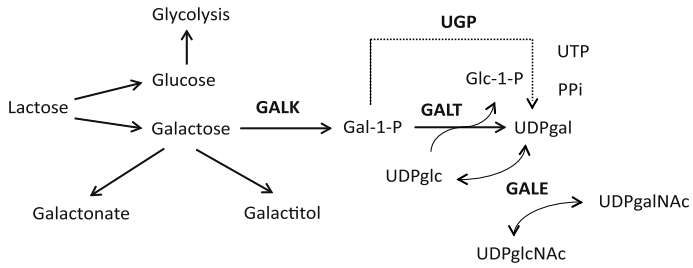
Generation of galt-deficient zebrafish

The design and assembly of the TALE nuclease (TALEN) construct targeting the *galt* gene (ENSDARG00000069543) to create a knockout was based on the protocol described by Cermak et al. (2011) (36). TALEN coding sequences were obtained from TAL Effector-Nucleotide Targeter (Bogdanove Laboratory, Cornell University; (36, 37)). In order to ensure loss-of-function of the galt protein, TALEN sequences located within the first part of the gene were chosen. The DNA binding sites for the TALEN pair targeting *galt* were: left site 5'-TTTTGGTCTCGGCCCATCGG-3', right site 5'-AAAGGACAGGTGGAGAAA-3'. Constructs were assembled using the Golden Gate TALEN and Tal Effector Kit 2.0 (Addgene) (36). The integrity of the final constructs was verified by Sanger sequencing.

The final TALEN plasmids were linearized by digestion with SacI. Afterwards, the DNA was purified and used as template for *in vitro* mRNA synthesis using the Ambion mMESSAGE mMACHINE T3 Transcription Kit (Life Technologies). The resulting mRNAs were purified by Lithium Chloride precipitation and integrity was verified using agarose gel electrophoresis.

Subsequently, the mRNA of both left and right TALEN constructs were injected in wildtype zebrafish embryos in one-cell stage. Injected fish were raised to adulthood and outcrossed, followed by confirmation of heterozygotes in the offspring. Heterozygous fish were incrossed in order to generate homozygous, *galt* knockout zebrafish (Figure 1; Supplementary Figures S1 and S2).

A



B

Human gene	Zebrafish gene	AA identity
GALM	*galm*	61%
GALK1	*galk1*	65%
GALT	***galt***	**73%**
GALE	*gale*	74%
AKR1B1	*akr1b1*	72%
UGP2	*ugp2a* *ugp2b*	83% 86%

Figure 1: Galactose metabolism in humans and zebrafish.
Genes encoding the enzymes involved in galactose metabolism (panel A) in humans show strong conservation with their zebrafish counterparts (panel B). Strikingly, only the *UGP2* gene is duplicated in zebrafish (*ugp2a* and *ugp2b*).
GALM: Galactose mutarotase, GALK1: galactokinase, GALT: Galactose-1-phosphate uridylyltransferase, GALE: UDP-galactose 4-epimerase, UGP: UDP-glucose/UDP-galactose pyrophosphorylase, AR: aldose reductase.
Panel B shows the percentage of amino acid similarity between the human and zebrafish homologues of the enzymes involved in galactose metabolism.

Genotyping

Zebrafish embryos were euthanized with a lethal dose of tricaine (MS222). Biopsies from the caudal fin of adult fish were removed with a sharp blade after anesthesia with MS222. Single embryos or fin clip biopsies were lysed in the same manner.

Embryos or fin clips were placed individually in 80 µl of lysis buffer, consisting of 1M KCl, 1M MgCl, 1M Tris pH 8.3, NP40, Tween-20 and gelatine, supplemented with Proteinase K to a concentration of 100 µg/ml. Lysis was performed for 1 h at 60 °C, 15 minutes at 95 °C and then held at 4°C. After adding 80 µl of ultrapure H_2O (Milli-Q, Merck Millipore) lysates were used for PCR.

PCRs encompassing the targeted region of *galt* were conducted using Phusion Hot Start II DNA Polymerase (Thermo Scientific) in 50 µl reactions with 5µl lysate per reaction as template. The following primers were used (including M13 sequence): forward

primer 5'-TGTAAAACGACGGCCAGTATCGTTTGAAGCCAAAATCG-3', reverse primer 5'-CAGGAAACAGCTATGACCTGCGTATTTCTCTGGATTTGC-3'. The PCR product was verified on a 1% agarose gel with ethidium bromide at a final concentration of 0.5 µg/ml.

15 µl of the PCR product were combined with 0.5 µl NcoI restriction enzyme. Reaction mixes were incubated at 37°C for three hours and then analyzed on a 3% agarose gel. Mismatches at the targeted region of *galt* were expected to result in loss of an NcoI restriction site, thereby generating two large fragments (526 and 345 bp) rather than three shorter fragments (408, 118 and 345 bp) (Figure 2). Presence of a mutation in only one *galt* allele will therefore lead to the presence of both wild type and mutant fragments (526, 408, 345 and 118 bp).

Mutations positive by NcoI digestion were confirmed by Sanger sequencing.

15 µl of PCR product were denatured and re-annealed in a thermocycler by incubation for 2 min at 95 °C, for 10 seconds at 85 °C, for 15 minutes at 25 °C and then held at 16 °C. The re-annealed products were treated with 0.5 µl of T7 endonuclease I (New England Biolabs) upon addition of 3 µl of buffer and 11.5 µl of H_2O (Milli-Q, Merck Millipore) to a final volume of 30 µl. Reaction mixes were incubated for 20 minutes at 37°C and analyzed on a 3% agarose gel. Mismatches at the targeted region of *galt* were expected to result in cleavage of heteroduplexes, thereby generating additional fragments.

Mutations positive by T7 endonuclease I were confirmed by Sanger sequencing.

To enable large-scaled genotyping, a High Resolution Melting (HRM) analysis was applied (38). Primers were designed using Primer3 software (39) (forward primer 5'-TACAATCCTCTGCGGGACTC-3'; reverse primer 5'-CGTGGGATGTTGTCCTCTG-3') and purified by high-performance liquid chromatography (HPLC). Amplification and HRM analysis were performed in the LightCycler ®480 (Roche Applied Science), using the High Resolution Melting Master kit (Roche Applied Science). Both genotype A and genotype B amplicons were analyzed according to the following conditions: 95°C for 10 minutes; 45 cycles of 95°C for 10 seconds, 59°C for 15 seconds, 72°C for 15 seconds; one cycle of 95°C for 60 seconds, 40°C for 60 seconds, and a melting from 60°C to 95°C rising at 0.02°C per second. All samples were run in triplicate. HRM analysis was performed using the software LightCycler ® 480 Gene Scanning version 1.5.1.

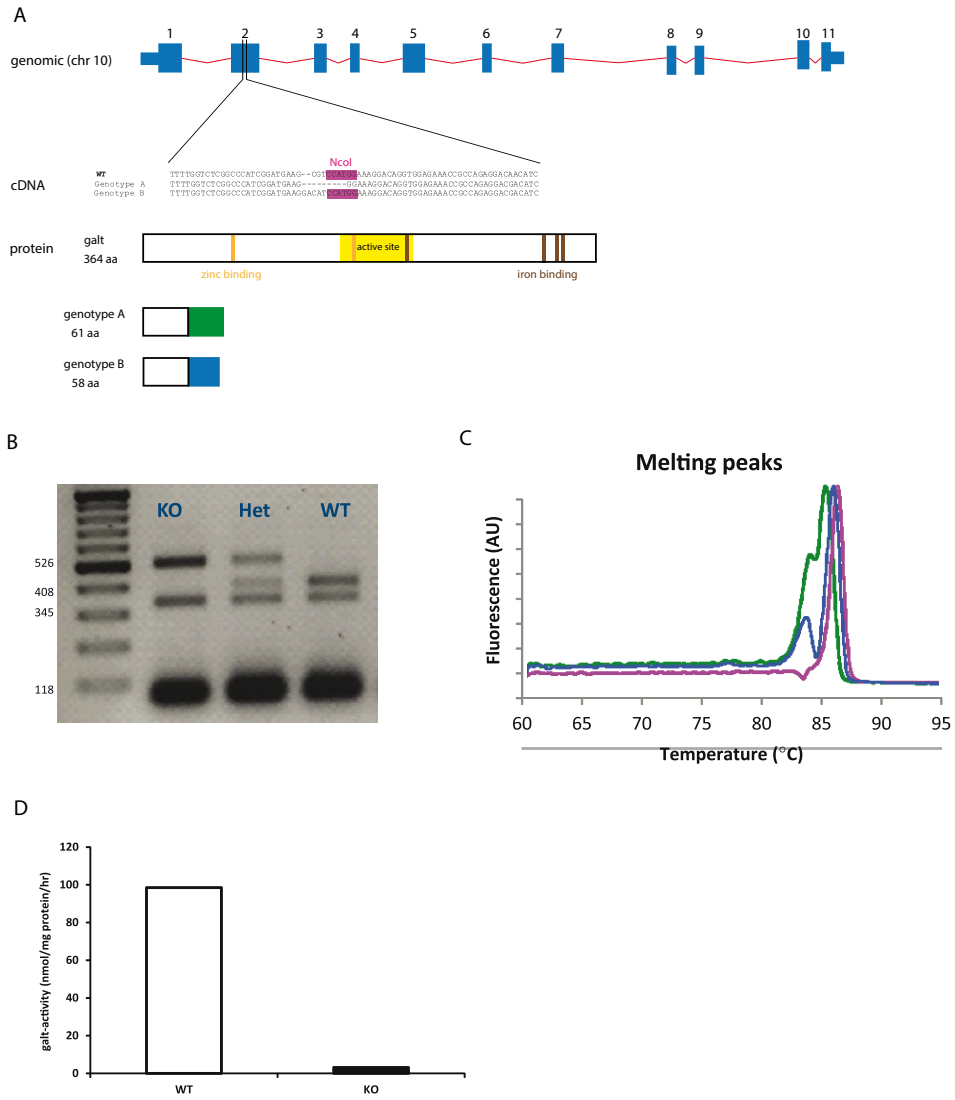


Figure 2: Establishment of a *galt* knockout model.
Panel A shows the genomic layout of the zebrafish *galt* gene. Injection of galt-specific TALEN constructs, targeting exon 2, resulted in two different mutations, both leading to a predicted frame-shift and a premature stop codon. The two genotypes of *galt* knockout zebrafish (genotype A and B) could be identified by NcoI, T7 endonuclease I and HRM analysis. Panel B illustrates a representative pattern of NcoI analysis of genotype A. Panel C illustrates a representative pattern for HRM analysis for genotype A or B (pink: wildtype, blue: heterozygous, green: knockout). Panel D shows a severe impairment of the galt catalytic activity in larval wildtype and knockout zebrafish (5dpf) using a HPLC assay.

Gene expression analysis RT-PCR

RNA was extracted from single adult organs using TRIzol (Life Technologies). A qScript cDNA Synthesis Kit (Quantabio) was used to generate cDNA. Quantitative real-time PCR (qPCR)-based quantification of *galt*, *gale* and *akr1b1* expression was conducted, with *ef1a* as a reference gene (40) . Primer sequences are available on request.. SYBR® green (Bioline) was used for all qPCR experiments. Samples, obtained from two independent RNA extractions were measured 5 times in total.

galt assay

In order to measure galt activity in zebrafish lysate with HPLC, 30 zebrafish embryos were suspended in 300 µl lysis buffer containing 80mM Tris pH 8.0 and cOmplete mini EDTA-free Protease Inhibitor (Roche). The suspension was homogenated in a potter tube (10 strokes) and subsequently sonicated by the ultrasonic processor UP50H (Hielscher) with a 2 mm diameter tip, amplitude 175 µm, power density 480W/cm^2. Lysates were centrifuged at 11.500 x *g* for 20 minutes at 4 °C. 50 µl of the supernatant, diluted to contain 30 µg of protein (assessed with BCA Protein Assay Kit [Thermo Scientific]), were mixed with 0.4 mM dithiothreitol, 30 mM Gal-1-P, 125 mM glycine and 15 mM UDP-glucose, and subsequently incubated for 60 minutes at 28 °C. The reaction mix was further processed and analyzed as described by Lindhout et al. (2010) (41). Moreover, two reaction mixes were used as controls, one containing 0.4 mM dithiothreitol, 125 mM glycine and 15 mM UDP-Glc (no Gal-1-P), and the other containing 0.4 mM dithiothreitol, 30 mM Gal-1-P and 125 mM glycine (no UDP-Glc).

Additionally, galt activity was confirmed through a liquid chromatography tandem mass spectrometry (LC-MS/MS) based assay by colleagues of Gerard T. Berry's laboratory, Boston Children's Hospital, Boston, USA **(42)**.

Zebrafish gale activity

Frozen tissues were added to a tube containing 0.5 mol/L glycine pH 8.5 (Sigma), 1% proteinase inhibitor cocktail (Sigma) and lysed by sonication at 4°C. Protein measurement, gale assay and LC-MS/MS settings were the same as previously described (43) except that 20µl of 4 mg/ml of brain and muscle lysate or 1mg/ml gonads lysate were added to the cocktail and incubated at 30°C for 30min. $^{13}C_3$-UDP-Glc was added as internal standard.

Zebrafish galt and ugp activity

For galt activity, the incubation medium consisted of 16µl of 0.5M glycine buffer pH 8.7, 8µl of 2mM UDP-Glc, 8µl of 2mM $^{13}C_6$-Gal-1-P and 8µl of 4 mg/ml tissue lysate and

incubated at 30°C water bath for 30min. Other steps were similar as described (42). The ugp activity was measured in a reaction mixture consisting of 4.35µl of 0.5M glycine buffer pH 8.7, 4.35µl of 0.66mM UTP, 4.35µl of 4mM $^{13}C_6$ Glc-1-P, 1.96µl of 6mM $MgCl_2$ and 5µl of 1 mg/ml tissue lysate and incubated at 30°C for 3 min. The reaction was stopped by adding 45µl of mixture composing of 7.8µl of 20µM $^{13}C_3$ UDP-Glc, 31.2µl of acetonitrile and 6µl of 0.6M formic acid. After mixing and centrifugation at 14000 x *g* for 10min, 30µl of the supernatant was used for LC-MS/MS analysis. Calibrator concentrations of UGP were 111, 37, 12.3, 4.1, 2.05 and 1.03 µmol/L.

All the samples were prepared in duplicate and measured twice.

Gal-1-P evaluation

In order to measure Gal-1-P in zebrafish, 30 zebrafish embryos were suspended in 300 µl lysis buffer containing 80mM Tris pH 8.0 and cOmplete mini EDTA-free Protease Inhibitor (Roche). The suspension was homogenized in a potter tube (10 strokes) and subsequently sonicated by the ultrasonic processor UP50H (Hielscher) with a 2 mm diameter tip, amplitude 175 µm, power density 480W/cm^2. Lysates were centrifuged at 11,500 x *g* for 20 minutes at 4 °C. Gal-1-P content was then determined in 70 µl of the supernatant, by a coupled-enzyme assay, as described in "Laboratory Guide to the Methods in Biochemical Genetics" (44). Briefly, in the first reaction, Gal-1-P is converted into galactose by alkaline phosphatase (AP), and in the second reaction, galactose is converted into galactone by galactose dehydrogenase (GDH). In the second reaction, NAD+ is used as a co-substrate by GDH, thus leading to the production of NADH, which is determined UV-spectrophotometrically(λ=340 nm).

Accordingly, Gal-1-P quantification was carried out using two mixes: the first containing 160 mM Tris pH 8.6, NAD^+ 12.5 mM, 43,000 U/ml AP and 60 U/ml GDH (galactose and Gal-1-P), and the second containing 160 mM Tris pH 8.6, NAD^+ 12.5 mM and 60 U/ml GDH (galactose). A mix containing no enzyme was also prepared and used as blank. Gal-1-P concentration was calculated by subtracting total galactose (determined from reaction 2) to total galactose and Gal-1-P (determined from reaction 1), and normalized to protein content. Total protein was quantified using the BCA Protein Assay Kit (Thermo Scientific) (45).

Development and survival evaluation

Development and survival of all fish was compared between genotypes from 5 dpf onwards until adulthood, in absence of exposure to galactose. At the age of 5 dpf morphology was studied with microscopy. Specific attention was paid to hatching and swim bladder development. Abnormal fish were genotyped to evaluate whether the three different genotypes (wildtype, heterozygous, knockout) were disproportionally present in these fish (i.e. whether the number of *galt* knockout fish was disproportionally high).

Assessment of survival rates, and comparison between genotypes, was conducted at a daily basis throughout the embryonic phase, and monthly thereafter.

Galactose challenge

Wildtype and knockout embryos (n = 30-50 per genotype) of 24 hpf were exposed to galactose-containing E3 medium (200 mM) in 100mm petri dishes. All animals were monitored daily for viability until they either died or reached 9 days of age.

Fertility evaluation

A selected pool of unchallenged, adult *galt* wildtype, heterozygous and knockout zebrafish (n = 60) was crossed periodically (once every 2-3 weeks, total of 9 crossings), to explore fertility in the different genotypes. The following crossings were conducted (six pairs per condition): wildtype incross, heterozygous incross, knockout incross, wildtype female crossed to knockout male, and knockout female crossed to wildtype male. The number of eggs and egg quality per spawning event per genotype were assessed.

Neurological assessment

Zebrafish embryos (5 dpf) and juveniles (4 weeks old) were placed in 24- and 12-well plates, respectively. Motor activity was quantified using a ZebraBox (Viewpoint) device under exposure to light for a period of 60 minutes. Activity is expressed as an average number of changes in pixels (arbitrary units) throughout the experiment. Motor activity was evaluated and compared between genotypes.

Statistical analyses

If required and appropriate, differences between two groups were evaluated for statistical significance using an independent t-test (normal distribution) or Mann-Whitney U test (no normality), both two-tailed, and differences between more than two groups were evaluated by an ANOVA test. For all tests a p value less than 0.05 was considered statistically significant.

RESULTS

Zebrafish metabolize galactose via the Leloir pathway

To explore the role of galactose metabolism in zebrafish, we first confirmed that the zebrafish genome encodes and expresses orthologues of the human genes encoding the Leloir pathway enzymes (GALK, GALT, GALE) and the enzymes involved in escape routes (AR, UGP) (Figure 1). A BLAST search identified a single zebrafish orthologue of human *GALT* on chromosome 10 of the zebrafish genome (ENSDARG00000069543). The zebrafish *galt* gene has two protein coding transcripts, of which the first (galt-001, ENSDART00000138161) encodes a protein of 364 amino acids and shows the strongest conservation with the human orthologue (73%) (Figure 1). Despite the fact that fish underwent a genome duplication throughout evolution (29), the *UGP2*-gene is the only duplicated gene related to galactose metabolism in zebrafish (Figure 1).

galt TALEN-generated mutants are loss-of-function

TALEN mRNA pairs targeting exon 2 of *galt* were injected in AB wildtype zebrafish embryos in the one-cell stage. A concentration of 150 pg of both TALEN mRNAs were injected after assessing toxicity and as a function of injection dose at 24 hpf. NcoI and T7 endonuclease I genotyping, followed by Sanger sequencing confirmation, revealed the generation of two *galt* mutant genotypes (Figure 2A and 2B). Genotype A (c.106_112delCGTCCAT) represents a 7 bp deletion, leading to a frame-shift at position c.106 and a subsequent introduction of a premature stop codon 26 amino acids downstream; the mutation is positive by NcoI and T7 endonuclease I analyses (Figure 2B). Genotype B (c.[106_107delCG; 106_109insGACA]) consists of a 2 bp deletion and 4 bp insertion, leading to a frame-shift at position c.106 and introduction of a premature stop codon 29 amino acids downstream; the mutation is negative by NcoI digestion and positive by T7 endonuclease I analysis. Genotype A and B presented the same melting profile by High Resolution Melting (HRM) analysis (Figure 2C).

A severe reduction of galt enzyme activity was measured in the knockout zebrafish embryos at 5dpf. (Figure 2D), confirming at the biochemical level that the knockout represents a loss-of-function allele.

Normal development and growth

The *galt* knockout fish undergo normal development and growth: hatching, swim bladder development or morphology are not affected throughout embryo, juvenile or adult stages. Thus, *galt* knockout zebrafish are morphologically indistinguishable from heterozygotes and wildtype fish; growth and survival rates are similar (data not shown).

galt, gale and ugp activity

The galt enzyme activity was undetectable in lysates of adult brain and gonads of galt knockout fish (Figure 3A and 3B). Catalytic activity of gale and ugp, two other enzymes of galactose metabolism, was also measured in lysates of adult brain and gonads, the target organs of long-term damage in classic galactosemia. The gale activity was marginally elevated in brain and slightly reduced in the gonads of knockout animals (not statistically significant). Overall, gale enzyme-activity was 6-7 fold higher in gonads as in brain, both in knockout and wildtype animals. Activity of ugp was also not significantly different in knockout versus wildtype tissues with no clear difference in activity in both tissues (Figure 3A and 3B). In addition, we assessed mRNA expression levels in adult brain and gonads of knockout animals vs. controls (Figure 3D).

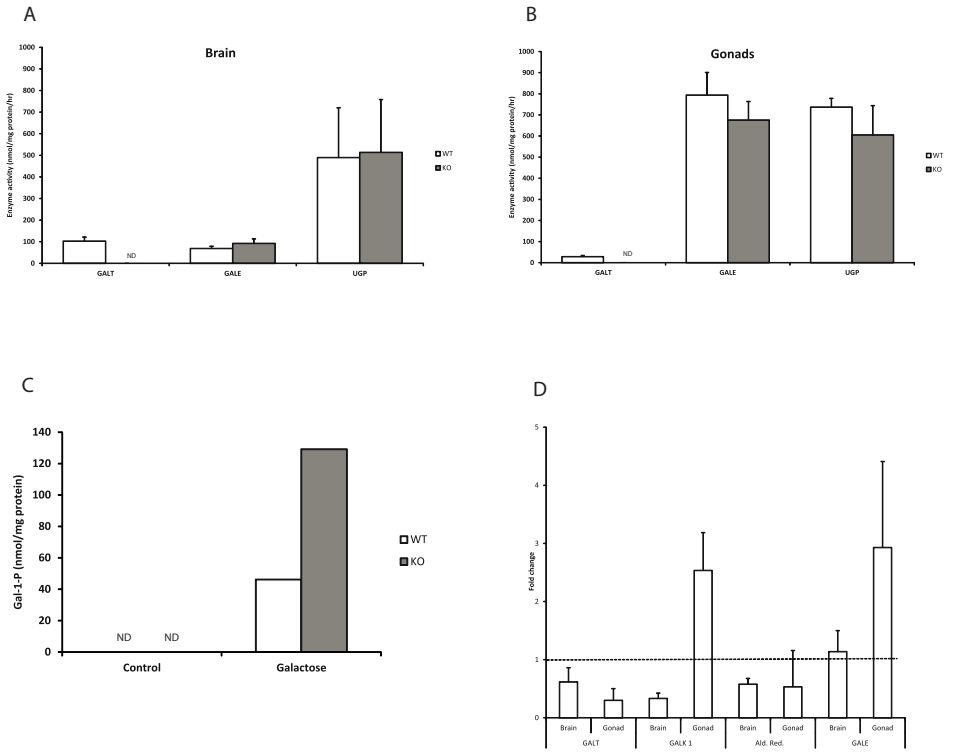


Figure 3: Biochemical phenotype of *galt* knockout zebrafish.
Panel A and B: *galt knockout* fish demonstrated essentially null galt activity in adult brain (panel A) and gonads (panel B) using an LC-MS/MS method. Enzyme activity of indicated enzymes is plotted per genotype as the average activity +/- SD. ND: not detectable.
When challenged with 200 mM of galactose for 8 days (1dpf-9dpf), knockout zebrafish accumulated high concentrations of Gal-1-P, whereas levels remained low in wildtype zebrafish (Figure 3C). ND: not detectable.
Panel D: mRNA expression levels of 4 key enzymes of the Leloir pathway was evaluated by QPCR. Values are expressed as fold change of expression of the knockout compared to the wildtype tissues.

galt knockout embryos accumulate Gal-1-P after exposure to galactose

To identify susceptibility to galactose, knockout embryos were challenged with different concentrations of galactose, of which a concentration of 200 mM galactose was chosen to proceed with the galactose challenge studies. In order to avoid inducing early developmental effects, embryos were exposed to galactose for 24 hpf onwards to the ages indicated.

When exposed to 200 mM of exogenous galactose, knockout embryos (5 dpf) accumulated significant amounts of Gal-1-P (259 nmol/mg protein) as compared to the unexposed situation (1.5 nmol/mg protein). In wildtype embryos, Gal-1-P levels did not differ between unexposed and exposed situations (0.29 and 0.28 nmol/mg protein, respectively). Comparable biochemical parameters were seen in embryos of 9 dpf. In absence of exogenous galactose, wildtype and *galt* knockout fish did not accumulate Gal-1-P (essentially null nmol/mg protein). In the challenged situation, *galt* knockout embryos accumulated much higher concentrations of Gal-1-P (129 nmol/mg protein) than wildtype embryos (46 nmol/mg protein).

Neurological assessment

Knockout embryos (5 dpf) showed similar activity as wildtype controls, both without (p = 0.472) and with (p = 0.114) prior exposure to exogenous galactose (data not shown). At a later stage, unchallenged 4 weeks' old knockout juvenile fish showed a statistically significant less activity than age-matched wildtype control (p = 0.002; Mann-Whitney U test) (Figure 4).

Fertility evaluation

Heterozygous pairs did not exhibit a statistically significant difference in egg quantity or quality as compared to wildtype pairs (p = 0.106 and p = 0.228, respectively).

Knockout pairs, wildtype female/knockout male and knockout female/wildtype male exhibited a lower egg quantity per mating as compared to wildtype pairs (Figure 4B).

The number of unsuccessful crossings revealed a higher frequency in the presence of a knockout female for knockout pairs and knockout female/wildtype male pairs, comparatively to wildtype pairs or to wildtype female/knockout male pairs (Figure 4C). Egg quality seemed to be not affected (Supplementary Figure S3).

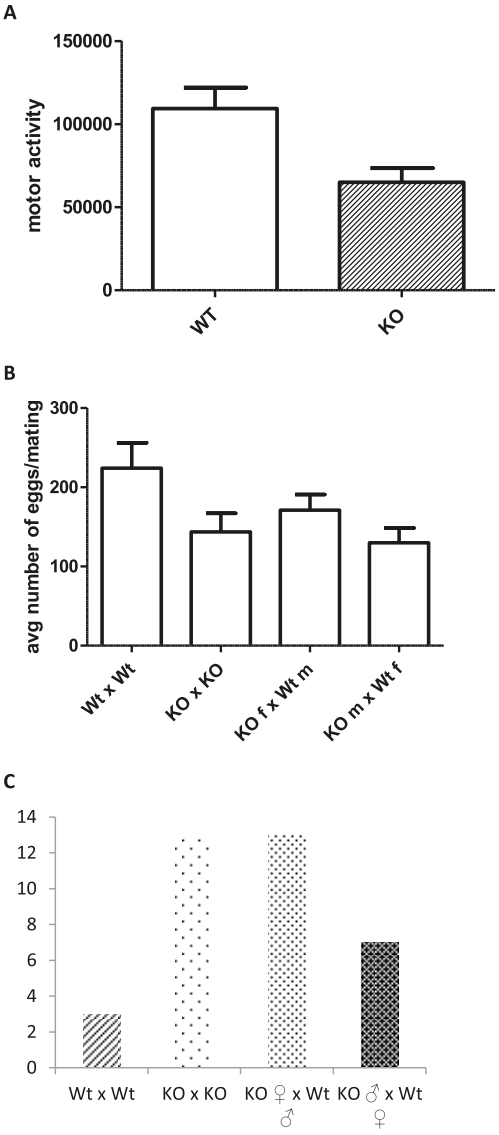


Figure 4: Chronic impairments.
Impairments of motor activity and fertility were observed in *galt* knockout zebrafish never exposed to exogenous galactose. Reduced motor activity was observed in knockout juvenile fish (4 weeks old) as compared to matched controls (n=66 per genotype; motor activity expressed as mean ± SEM) (panel A). Adult knockout pairs, wildtype female/knockout male and knockout female/wildtype male exhibited a lower average egg quantity per mating as compared to wildtype pairs (panel B; expressed as mean ± SEM). Knockout pairs demonstrated a premature decline in egg quantity as compared to wildtype pairs (panel C). The number of unsuccessful crossings was higher in the presence of a knockout female (panel D).

DISCUSSION

In the current study, we described the successful development and initial characterization of a *galt* knockout zebrafish model for classic galactosemia. During the past years, two other disease models were generated, a fruit fly model and a mouse model (18, 24). We created our zebrafish line complementary to these models, since the many advantageous features of the zebrafish allow novel investigations that can further improve our understanding and management of this disorder.

The *galt* knockout zebrafish mimics the human phenotype at the biochemical and clinical levels. Enzymatic assays confirmed successful loss-of-function, with essentially null galt activity.

Knockout fish accumulated elevated concentrations of Gal-1-P upon exposure to exogenous galactose. At juvenile stage, without prior exposure to exogenous galactose, *galt* knockout fish showed a significantly decreased motor activity as compared to wildtype fish. Furthermore, a premature decline of reproductive capacity in adult knockout zebrafish without prior exposure to exogenous galactose as compared to wildtypes was demonstrated. Knockout female fish presented a higher frequency of unsuccessful crossings. The decrease in motor activity might influence mating behavior and indirectly affect the reproductive capacity.

Catalytic activity of gale and ugp were also measured, and interestingly varied considerably in the various tissues, which probably reflect specific needs of the given organ (30).

These findings are in line with the biochemical and clinical phenotype of patients with classic galactosemia, who develop acute toxicity in the newborn phase when exposed to galactose-containing milk (31). At a biochemical level, patients accumulate high levels of gal-1-P in the newborn phase upon exposure to galactose (13). Chronic impairments of brain, ovaries and, to a lesser extent, bones occur despite adequate compliance to the diet, and even those individuals who were never exposed to galactose as a newborn are affected (32-34). The observation of impaired motor activity in unexposed *galt* knockout zebrafish at juvenile stage seems to be in line with the human phenotype of neurological sequelae (9). Furthermore, unexposed adult knockout fish presented a decreased reproductive capacity. Knockout females exhibited an increased frequency of unsuccessful crossings, and the presence of a mutant allele was associated with a lower egg quantity per mating. These findings are in line with the primary ovarian insufficiency (POI) seen in nearly all females with classic galactosemia (11) and with previous reports of a possible male fertility impairment. Moreover, the clinical findings in our zebrafish model are in line with observations in other animal models for classic galactosemia. GALT-deficient flies exhibited motor impairments (24), whereas GALT-deficient mice exhibited brain abnormalities, and females showed a smaller litter size and longer time to achieve pregnancy (18).

With the creation of our zebrafish line, we have generated a model to study organ development from embryo stage to adulthood, which can provide important new in-

sights on when lesions of target organs occur in this disease. Hitherto, it remains to be elucidated whether organ toxicity has a prenatal or postnatal onset, or extends over multiple developmental stages. Though the *Drosophila melanogaster* model and mouse model already demonstrated great importance for pathogenesis studies, the zebrafish model is more amenable to organ studies throughout development, which are essential to answer the long-standing open question if, and to what extent, prenatal organ damage occurs. The external development of the zebrafish, the availability of transgenic reporter lines and the accessibility to sophisticated imaging techniques make our model an outstanding tool for studies of folliculogenesis and myelogenesis from embryonic stage to adulthood. In addition, the high-throughput screening potential of our model facilitates rapid and efficient testing of pharmacologic compounds, thereby contributing to the development of new therapeutic approaches.

In conclusion, we have successfully developed a *galt* knockout zebrafish model for classic galactosemia, which recapitulates the human phenotype at biochemical and clinical levels. We generated this model because of the unique features of the zebrafish that allow organ studies throughout development and high throughput screening of pharmacological compounds, thereby underlining the importance of this model as a complement to the existing models. Future studies in our model will contribute to improved understanding and management of this enigmatic disorder.

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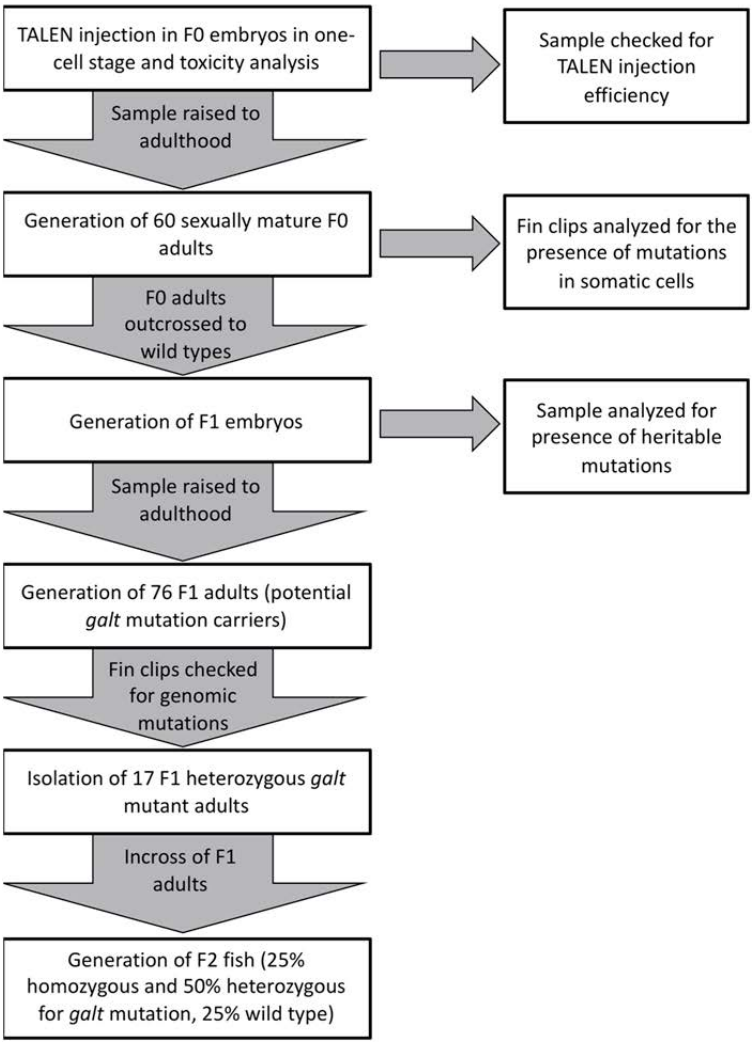


Figure S1: Flowchart for the generation of a *galt* knockout zebrafish line.
The steps involved in the establishment of a *galt* knockout zebrafish line are outlined.

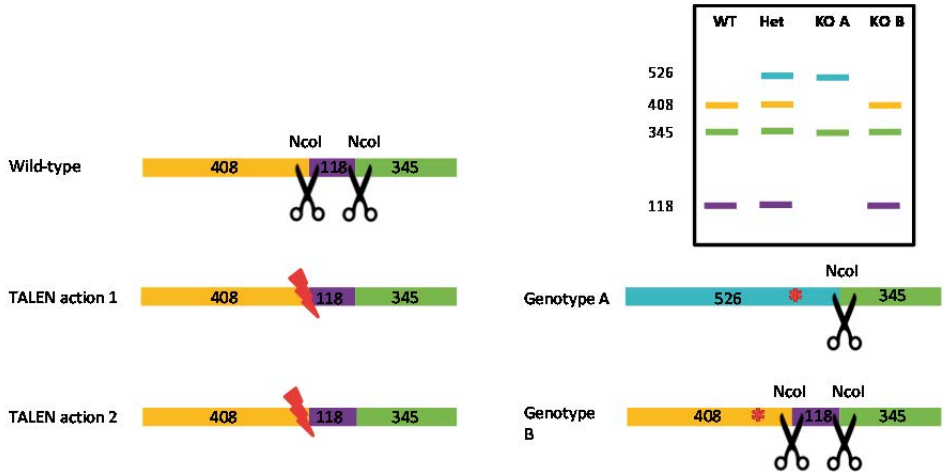
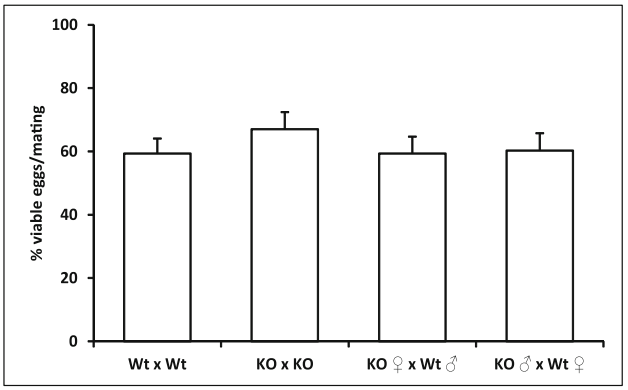


Figure S2: Fragments generated after NcoI digestion in galt wildtype, heterozygous and knockout zebrafish. TALEN construct targeting exon 2 of the *galt* gene (red lightning bold) has two distinct modes of action, thereby generating two different mutations. Genotype A: a TALEN-derived mutation (red asterisk) at the position of an NcoI restriction site (black scissors), which results in loss of this restriction site and thus an aberrant digestion pattern. Genotype B: a TALEN-derived mutation before the NcoI restriction site, which does not affect the digestion pattern and is thus only rapidly detectable through T7 endonuclease I analysis. Both genotypes result in a frame-shift and subsequently a premature stop codon. As a result, wildtype (WT), heterozygous (Het) and knockout (KO) fish demonstrate different digestion patterns.



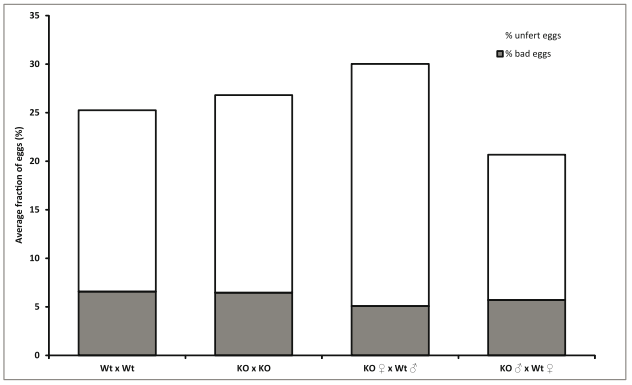


Figure S3: Evaluation of egg-quality during subsequent mating events.
Next to quantity of eggs in successive spawning events, also egg quality was assessed. This was performed by quantifying the amount of viable and unviable eggs immediately after spawning (measure for bad egg quality) and at 24hpf to evaluate fertilization problems.

Chapter 3

Fertility in adult women with classic galactosemia and primary ovarian insufficiency

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Submitted

ABSTRACT

Objective: To study pregnancy chance in adult women with classic galactosemia and primary ovarian insufficiency. Despite dietary treatment, over 90% of females with classic galactosemia develop primary ovarian insufficiency, resulting in impaired fertility. For many years, chance of spontaneous conception has not been considered, leading to counseling for infertility. Yet, the increasing number of reports on pregnancies in this group questions whether current counseling approaches are correct.

Design: Multicenter observational study.

Setting: This study was conducted in fifteen metabolic centers in the Netherlands, Austria, Belgium, Estonia, France, Ireland, Spain, Switzerland, and the United States of America.

Patients: Adult females (aged ≥18 years) with confirmed classic galactosemia and primary ovarian insufficiency were included.

Intervention(s): Participants and medical records were consulted to obtain study data in a standardized manner using a questionnaire.

Main Outcome Measure(s): Conception opportunities, time to pregnancy, pregnancy outcome, hormone replacement therapy use, fertility counseling, and the participants' vision on fertility were evaluated. Potential predictive factors for increased pregnancy chance were explored.

Results: 85 women with classic galactosemia and primary ovarian insufficiency participated. 21 women actively attempted to conceive or did not take adequate contraceptive precautions. Of these 21 women, nine became pregnant spontaneously (42.9%). This was significantly higher than reported in primary ovarian insufficiency due to other causes ($p < 0.0001$, 1-sided). After a period of 12 months, a cumulative proportion of 27.8% of couples had conceived, which increased to 48.4% after 24 months and 61.3% after 27 months. Predictive factors could not be identified. A considerable miscarriage rate of 30% was observed (6/20 pregnancies). Though a substantial proportion of women expressed a child-wish (n=28/53, 52.8%), the vast majority of participants (n=43/57, 75.4%) considered conceiving highly unlikely, due to negative counseling in the past.

Conclusion: The pregnancy rate in women with classic galactosemia and primary ovarian insufficiency was higher than expected. This shifting paradigm carries significant implications for fertility counseling and potential application of fertility preservation techniques.

INTRODUCTION

Primary ovarian insufficiency (POI) comprises a continuum of impaired ovarian function and is an important cause of menstrual cycle disorders and fertility impairments in women (1). With its prevalence of over 90% in females with classic galactosemia (2), it represents the most frequent long-term complication of this inborn error of galactose metabolism. Galactose is a sugar highly important for energy delivery and glycosylation purposes, among other functions (3). The Leloir pathway constitutes the main route for galactose degradation, with one of the key enzymes being galactose-1-phosphate uridylyltransferase (GALT). Severe deficiency of this enzyme, due to mutations of the *GALT* gene, results in the metabolic disorder known as classic galactosemia, affecting approximately 1 in 16,000-50,000 newborns (4, 5). The disease is characterized by neonatal onset of a toxic syndrome that quickly resolves when galactose-restriction is initiated. However, despite strict dietary treatment, nearly all females develop POI with consequent reduced fertility.

The remarkably high prevalence of hypergonadotropic hypogonadism in women with classic galactosemia was first reported in 1979 (6). Meanwhile, evolved experience has shown that female patients exhibit a wide phenotypic spectrum of POI, varying from young adolescents with primary amenorrhea to individuals with normal pubertal development but irregular or absent menstrual cycles at a later stage. There seems to be a more rapid decline in the number of primordial follicles in prenatal or early postnatal life (7). Several pathophysiological mechanisms have been postulated, including toxicity of galactose and its metabolites to primordial germ cells, oocytes, and follicles (8), signaling pathway abnormalities (9), and aberrant glycosylation (10) resulting in, among others, leptin dysregulation (11). The hypothesis of a prenatal or early postnatal origin of the ovarian damage is supported by the consistently reduced concentrations of anti-Müllerian hormone and elevated gonadotropin concentrations that can be found in these girls already at an early age (12, 13).

Due to the varying and unpredictable course of impaired ovarian function that POI reflects, pregnancies are not excluded in women with this diagnosis (1), and in general 5-10% of women with POI of any cause conceive spontaneously (14). Also in women with classic galactosemia spontaneous pregnancies have been reported regularly despite the presence of POI (summarized in (15), (4, 5, 16-19)). Thus far only one study in a small number of patients (n=22) addressed pregnancy attempts in females with classic galactosemia and concluded that most females did not try to become pregnant because they had been told they could not have children (15). Interestingly, of the few women who attempted to conceive spontaneously (n=9), as much as 4 (4/9 = 44%) succeeded.

These data lead to the question whether pregnancy chance for this population might be higher than for women with POI due to other causes. Further exploration of pregnancy chance in this specific group is crucial to improve counseling of young patients and their parents. Furthermore, increased insight is essential for accurate as-

sessment of the potential value of fertility preservation techniques (20). This international epidemiological study investigates fertility in a cohort of adult women with classic galactosemia and POI, in order to determine whether current reproduction counseling of patients and their families is accurate.

METHODS

Study design and participants

This observational study was conducted in fifteen metabolic centers in the Netherlands, Austria, Belgium, Estonia, France, Ireland, Spain, Switzerland, and the United States of America (USA). Centers and patients were informed on this study through the international Galactosemia Network (http://www.galactosemianetwork.org/) and patient associations, respectively. Patients' participation and informed consent were solicited during gatherings of patient associations, at the outpatient clinic, or by phone in case a patient had participated in an earlier study and had given consent to be recontacted. Participants were enrolled between February 2014 and July 2016. We obtained ethics approval for this study from local research ethics committees in participating centers in the different countries. Informed consent was obtained.

Adult females (≥18 years of age) with classic galactosemia and POI were invited to participate in the study. Classic galactosemia diagnosis was confirmed by GALT enzyme activity assay and/or *GALT* mutation analysis. POI was defined as impaired ovarian function (primary or secondary amenorrhea, menstrual interval of >90 days, or oligomenorrhea of <9 cycles/year (21)) before the age of 40 years, supported by two serum follicle-stimulating hormone (FSH) concentrations (obtained at least 1 month apart) in the menopausal range (1).

Study procedure and outcome measures

Patients and medical records were consulted to collect study data in a standardized manner using a questionnaire, translated in different languages (see supplemental material for English version), developed, validated and used by our group in the past (15). Information on GALT enzyme activity, *GALT* mutation, age at diet initiation, estimated galactose intake (<20, 20-200 or >200 mg/day (22)), educational level (classified according to International Standard Classification of Education [ISCED] 2011 guidelines (23)), puberty development, menstrual history, ovarian function (i.e. serum FSH/LH/estradiol concentrations, results of ovarian imaging), and hormone replacement therapy (HRT) use were obtained. Additionally, pregnancy opportunities in the past (defined as periods of regular sexual intercourse without adequate precautions to avoid conception), time to pregnancy, and the number of spontaneous conceptions and their

outcomes were assessed. Furthermore, fertility counseling and the patients' own visions about fertility and future pregnancies were evaluated.

Statistical analysis

The quantitative variables were reported through means and standard deviations (or by medians and ranges if non-normally distributed, as assessed by Shapiro-Wilk test). The qualitative variables of the questionnaire were reported through frequency-analysis and percentages. Characteristics of patient subgroups were compared using either a Mann-Whitney U test in case of numeric variables or a chi square test/Fisher's exact test in case of categorical variables. Potential predictive factors were formulated prior to start of the study (genotype p.Q188R/p.Q188R, GALT enzyme activity <0.4%, spontaneous menarche, low educational level, and galactose intake of >20 mg/day), based on previous literature (13, 15, 22, 24, 25), and subsequently evaluated using Fisher's exact test and odds ratios (OR). The proportion of females in our cohort that became pregnant was compared to the 5-10% in women with POI in general using a binomial test. Time to first pregnancy was evaluated using a survival analysis and presented in a Kaplan Meier curve. Based on a sample size calculation with a desired power of 80%, an estimated effect of 24-44% in the classic galactosemia group and an estimated effect of 7.5% in the reference group (14), inclusion of a minimum of 9-27 women that had tried to conceive was aimed for (26). All data analyses were performed by SPSS version 23. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Descriptive characteristics of the total cohort (n=85) and the cohort stratified by pregnancy opportunities are presented in Table 1. Overall, the median age in the cohort was 30 years (range 18-53 years). The most prevalent genotype was p.Q188R/p.Q188R (n=45/65, 69.2%) and median GALT enzyme activity was 0.3% (range 0.0-1.2%, n=25). Galactose-restriction was initiated at a median age of 10 days (range 0-2920 days, n=75). Current galactose intake was restricted in the vast majority of patients (estimated galactose intake <20 mg/day; n=72/84, 85.7%); only some adhered to a diet with small relaxations (estimated galactose intake 20-200 mg/day; n=7/84, 8.3%) or a more relaxed diet (estimated galactose intake >200 mg/day; n=5/84, 6.0%). The level of education in most patients was low (ISCED 2011 level 0-2; n=26/67, 38.8%) or intermediate (ISCED 2011 level 3-4, n=31/67, 46.3%).

Of the total cohort, 47 women (n=47/85, 55.3%) presented with primary amenorrhea (induced menarche) and 38 (n=38/85, 44.7%) had a spontaneous menarche. First menstruation (either spontaneously or after induction with exogenous estrogen) occurred at a median age of 15 years (range 10-28 years, n=78). The majority of partici-

pants were using some form of HRT (n=59/75, 78.7%); the birth control pill was the most commonly used type (n=32/75, 42.7%).

Women who did not experience pregnancy opportunities (i.e. women who had taken contraceptive precautions to avoid conception or had never been sexually active) were younger ($p < 0.0001$) and were more often on a strict diet (<20 mg/day) ($p < 0.01$) than those who had pregnancy opportunities (i.e. women who actively attempted to conceive or had regular unprotected sexual intercourse). Also, induced puberty and HRT use were more frequent in the group of women without pregnancy opportunities, which could partly reflect improved clinical awareness. Yet, these group differences might also result from a less severe phenotype in the group of women with pregnancy opportunities, despite high similarity in genotype, GALT activity, diet onset, and cognitive functioning.

Table 1: Patient characteristics.
Demographics of the complete cohort (n=85) are presented. Furthermore, characteristics of participants stratified by information on pregnancy opportunities (yes/no, n=71) are shown. Variables that significantly differ between these two groups are marked with an asterisk.

	Total group (n=85)	Without conception opportunity (n=50)	With conception opportunity (n=21)
Age [1]	30 (18 - 53; n=85)	24 (18 - 44; n=50)	33 (24 - 53; n=21) *
Genotype p.Q188R/p.Q188R	69.2% (n=45/65)	65.7% (n=23/35)	62.5% (n=10/16)
GALT activity [2]	0.3 (0.0 - 1.2; n=25)	0.3 (0.0 - 1.2; n=20)	0.3 (0.0 - 1.0; n=5)
Onset diet [3]	10 (0 - 2920; n=75)	8 (0 - 2920; n=48)	13 (0 - 195; n=18)
Strict diet [4]	85.7% (n=72/84)	92.0% (n=46/50)	65.0% (n=13/20) **
Low education [5]	38.8% (n=26/67)	43.2% (n=19/44)	35.3% (n=6/17)
Spontaneous menarche	44.7% (n=38/85)	42.0% (n=21/50)	71.4% (n=15/21) ***
Age at first menstruation [6]	15 (10 – 28; n=78)	14.5 (10 – 20; n=48)	15 (12 – 20; n=20)
HRT [7]	78.7% (n=59/75)	83.3% (n=40/48)	55.6% (n=10/18) ****
Child-wish	52.8% (n=28/53)	47.5% (n=19/40)	66.7% (n=8/12)
Negative counseling [8]	75.4% (n=43/57)	72.5% (n=29/40)	87.5% (n=14/16)

[1] Median and range of age at time of participation in years
[2] Median and range of residual galactose-1-phosphate uridylyltransferase (GALT) activity in percentage of control values
[3] Median and range of age at initiation of dietary management in days
[4] A strict diet is defined as a galactose intake of <20 mg/day (22)
[5] A low educational level is defined as International Standard Classification of Education 2011 level 0-2 (23)
[6] Median and range of age at first menstruation in years
[7] Hormone replacement therapy (HRT) at time of participation
[8] Patient thinks pregnancy is impossible, based on fertility counseling by healthcare professionals
* $p < 0.0001$
** $p < 0.01$
*** $p = 0.04$
**** $p = 0.03$

Only 21 women (n=21/71, 29.6%) had actively attempted to conceive spontaneously or had experienced a period of regular unprotected sexual intercourse, and of these 21 women nine became pregnant (n=9/21, 42.9%). A binomial test indicated that this proportion of pregnancies of 42.9% was significantly higher than the expected proportion of 7.5% (14) ($p < 0.0001$, 1-sided). A Kaplan Meier curve (n=16 cases, of which seven conceptions) demonstrated that after a period of 12 months 27.8% of couples had conceived (estimated cumulative proportion; n=4), which increased to 48.4% after 24 months (n=6) and 61.3% after 27 months (n=7) (Figure 1). A total of 20 spontaneous pregnancies in nine women were reported in this cohort (three women conceived four times, two females conceived twice and the other four women conceived once), of which six ended in a miscarriage (in five individuals; n= 6/20, 30.0%)). Women who conceived did not differ from women who could not conceive with regard to characteristics (Table 2). Also, age ≤25 years at first pregnancy opportunity ($p = 0.34$, OR: 3.50 [0.43 - 28.45]), genotype p.Q188R/p.Q188R ($p = 1.00$, OR: 1.00 [0.13 - 7.57]), history of a spontaneous menarche ($p = 0.66$, OR: 1.75 [0.24 - 12.64]), estimated galactose intake of <20 mg/day ($p = 1.00$, OR: 0.83 [0.13 - 5.40]), and low educational level ($p = 0.16$, OR: 5.33 [0.62 - 45.99]) did not demonstrate significant effects on the occurrence of spontaneous conception.

Table 2: Characteristics of women who did not conceive despite pregnancy opportunities (n=12/21) and women who conceived (n=9/21).
None of the variables was significantly different (p <0.05) between the two groups.

	Did not conceive (n=12)	Conceived (n=9)
Age at first opportunity [1]	30 (25 - 32; n=9)	24 (21 - 38; n=8)
Genotype p.Q188R/p.Q188R	62.5% (n=5/8)	62.5% (n=5/8)
Onset diet [2]	13 (0 - 195; n=11)	13 (5 - 32; n=7)
Strict diet [3]	66.7% (n=8/12)	62.5% (n=5/8)
Low education [4]	20.0% (n=2/10)	57.1% (n=4/7)
Spontaneous menarche	66.7% (n=8/12)	77.8% (n=7/9)
Age at menarche [5]	16 (12 - 20; n=11)	14 (13 - 17; n=9)
Negative counseling [6]	90.9% (n=10/11)	80.0% (n=4/5)

[1] Median age (range) in years at time of first pregnancy opportunity.
[2] Median and range of age at initiation of dietary management in days
[3] Strict diet is defined as a galactose intake of <20 mg/day (22)
[4] Low educational level is defined as International Standard Classification of Education (ISCED) 2011 (23) level 0-2
[5] Median and range of age at first menstruation in years
[6] Patient thinks pregnancy is impossible, based on fertility counseling by healthcare professionals
Note: Residual galactose-1-phosphate uridylyltransferase (GALT) activity was discarded as a variable due to inadequate sample size (n=5). Data on women who conceived (n=3) and women who did not conceive (n=2) showed a residual GALT activity ≤1.0% in all females.

About half of the women expressed a child-wish, now or in the future (n=28/53, 52.8%) (Table 1). However, the majority of participants (n=43/57, 75.4%) reported healthcare professionals told them that pregnancy was not feasible, and that they therefore considered pregnancy highly unlikely. Interestingly, one participant did not even try to become pregnant spontaneously, despite an active child-wish, because she believed she would not succeed. She and her partner decided to proceed to oocyte donation directly, and the woman is now carrying her second child (both pregnancies established with donor oocytes). The other half of participants had no intention of ever having children, and the most important reason for this was that many females considered themselves unable to bring up a child. Four out of nine women became pregnant unplanned, under the assumption contraceptives were not needed.

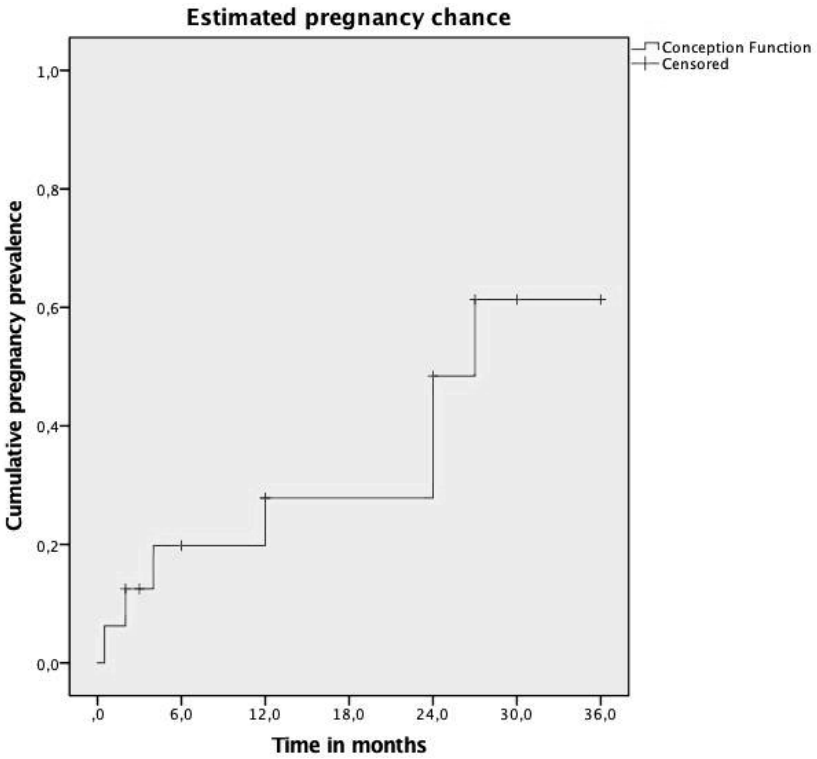

Figure 1: Kaplan Meier curve of conception in study cohort. Function for conception is presented, with duration of pregnancy opportunity (i.e. time period wherein no precautions were taken to prevent conception) in months on X-axis and cumulative pregnancy prevalence on Y-axis. Couples that did not conceive during their period of pregnancy opportunities are censored (tick marks). Only outcomes of subjects' first pregnancy opportunities are included.

DISCUSSION

In this observational study among women with classic galactosemia and POI, our data indicate that fertility is not as impaired as previously thought, which has significant implications for counseling of these patients and their families regarding reproduction and fertility preservation.

Studies in the general population indicate that 70% of couples achieve pregnancy within a period of 6 months of unprotected sexual intercourse, which increases to a proportion of 80% after 12 months and 90% after 24 months (27). Our results indicate that chances for women with classic galactosemia and POI are substantially lower, namely 20%, 28% and 48% after 6, 12, and 24 months, respectively (Figure 1). This is not unexpected, given that these females have considerable ovarian damage and a severely compromised ovarian reserve. However, if these women attempt to conceive for a slightly longer period of time (27-30 months), over 60 percent of couples are estimated to conceive. Previous studies reported that in general 5-10% of women conceive spontaneously after POI diagnosis (14). We observed a significantly higher spontaneous pregnancy prevalence of 43% in our study cohort ($p < 0.0001$).

Albeit time to pregnancy is expected to be longer than for women with an unaffected ovarian reserve, many females with classic galactosemia do not try to conceive for as long as 1 year, let alone 2 years (Figure 1). An explanation for this premature abandonment of trying to conceive given by many women is the discouraging nature of fertility counseling by healthcare professionals, which is reported by three quarters of participants. Since women consider pregnancy highly unlikely, several months of unsuccessful attempts might strengthen these thoughts and make them give up early.

Our study emphasizes the importance of adequate precautions to avoid conception, in case pregnancy is not desired, despite POI diagnosis. We showed that half of patients do not have the intention of ever having children and that several women became pregnant unplanned. The need for contraception in those cases should be underlined in counseling.

It remains unclear why some women conceive spontaneously and others fail to become pregnant. We explored several characteristics with a potentially predictive value. Though the p.Q188R/p.Q188R genotype was reported to be a risk factor for POI development (24), our data indicate that pregnancy chance of women with this genotype is not lower than for patients with other classic *GALT* mutations (OR: 1.00, 95% CI: 0.13 - 7.57). Moreover, spontaneous menarche was suggested to result in increased chance of spontaneous pregnancy (15), yet our findings do not fully support this hypothesis. We observed a higher frequency of spontaneous menarche in women who had pregnancy opportunities as compared to women who did not have pregnancy opportunities, which might reflect a less severe phenotype in this group due to other factors than genotype, GALT activity, and diet onset. Within this subgroup, we did not observe differences with regard to menarche between women who became pregnant and women who could not

have children (OR: 1.75, 95% CI: 0.24 - 12.64). We were unable to evaluate the potential effect of residual GALT enzyme activity ≥0.4% on pregnancy chance due to the limited number of patients for whom this data was available (13). Also, low educational level and a strict diet did not show significant effects, though findings could result from the limited sample size.

In this cohort, a total of 20 spontaneous pregnancies were observed, of which six ended in a clinically recognized miscarriage (30%). This is relatively high as compared to the general population, in which 10-15% of all pregnancies is estimated to end in a clinically recognized spontaneous abortion (28).

Furthermore, the current data challenge the application of invasive fertility preservation techniques at an early age. As reported previously, fertility preservation in this population is only likely to be successful in very young girls, due to the severely depleted follicle pool from an early age on (20), which is supported by the poor response to ovarian stimulation in the majority of adult patients (7). Cryopreservation of ovarian tissue is the only available technique at prepubertal age. Though experience with this method has evolved, tissue quality and pregnancy chances remain only limitedly known (29, 30). Thus far, there are no reports on transplantation of cryopreserved tissue in classic galactosemia, so the potential impact of the disease's (ongoing) pathogenesis on the quality of the frozen tissue and its follicles is yet to be determined. Furthermore, application of this technique might reduce future chances of spontaneous pregnancy by removal of one entire ovary.

This is the largest cohort hitherto studied to estimate pregnancy chance of women with classic galactosemia and POI. Despite our international multicenter study design and major efforts to include as many participants as possible, conclusions on pregnancy chance derive from a small subgroup (n=21), since only a limited number of participants had conception opportunities in the past. This could partly be related to the relatively low median age in the cohort (30 years). The limited subgroup size, as well as the potential selection bias of women with successful pregnancy attempts (we experienced that women who did not succeed in conceiving sometimes do not want to participate because of painful memories), could influence the pregnancy prevalence that was observed in our study.

In conclusion, the pregnancy rate in women with classic galactosemia and POI was higher than expected, even though many women did not try to conceive because they were told they are infertile. If more women would attempt to conceive, and if they would try for a longer period of time than they generally do now (>2 years), potentially more pregnancies would occur in this group. Importantly, there was a substantial risk of miscarriage in this cohort. Our data corroborate the paradigm that women with classic galactosemia have a considerable chance of spontaneous pregnancy despite POI. These findings prompt revision of current fertility counseling and the potential application of fertility preservation.

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SUPPLEMENTAL MATERIAL

Questionnaire pregnancy chance in classic galactosemia

Personal data

1 What is your age? …….. years
2 In which country were you born? ……
Where does your family come from? ……
3 What is your height? …….. cm or feet/inches
4 What is your current weight? …….. kg or pounds
5 What is/was your job? ……
6 What is your highest level of education? ……
7 Do any diseases run in your family? **No / Yes**, ……
8 Does any of your family members suffer from infertility? **No / Yes**
Which family member(s) suffer(s) from infertility and which disease causes his/her/their infertility?
……

Medical history

9 Are you currently under treatment of a doctor? **No / Yes**,
Name: ……
Specialty: ……
Reason: ……
10 Have you ever undergone surgery? **No / Yes**, for: ……
Year: ……
11 Were you ever treated by a doctor for another reason? **No / Yes**, for:
Name: ……
Specialty: ……
Reason: ……
Year: ……
12 Do you use any medication? **No / Yes**, I use ……
13 Do you smoke? **No / Yes**, …… cigarettes per day
14 Do you drink alcohol? **No / Yes**, …… glasses per week
15 Do you drink coffee or tea? **No / Yes**, …… cups coffee and …. cups tea per day

Classic galactosemia

16 How old were you when doctors found out that you have classic galactosemia?
……years
17 Are you on a (ga)lactose restricted diet? **No / Yes**
If you are not on a diet, have you ever been on a diet? **No / Yes,** from
…… until ……
If you are on a diet, since when? **Since** ……

Gynecological history

18 At what age did you experience your first period? ..... years
19 Did your need help to get your periods started? **No / Yes,** it was started with medication called ……
20 Are your periods regular? **Yes / No**, cycle: ……..
Have your periods always been regular? **Yes / No**, cycle: ……..
21 Do/did you use oral contraceptives (the pill)? **No / Yes,** name: ……..
When? From ……. until ……..
22 Do you have (biological) children? **No** *(continue to question 26)* **/ Yes**
How many? …… children
23 Have you ever had a miscarriage? **No / Yes,** …. miscarriages at …… weeks of pregnancy
24 How long did it take you and your partner to become pregnant? ……months
25 Did you need help from a doctor (medical assistance, assisted reproductive techniques) in order to become pregnant? **No / Yes,** I needed…….. …… *Continue to question 29*

26 Have you ever tried to become pregnant? **No** *(continue to question 29)* **/ Yes**
27 How long did you and your partner try to become pregnant?
..... months
Period: ……..until…..
Your partner's age at the time: …….years
28 Have you had a miscarriage?
No / Yes, ….. miscarriages at …… weeks of pregnancy

29 Have you ever undergone examinations or tests to determine whether you are fertile? **No / Yes,** the result was …….
Have you been diagnosed with primary ovarian insufficiency or premature ovarian failure? **No / Yes**, based on: **Ultrasound / Hormone levels in blood / Other,**……..
30 Has your partner ever undergone examinations or tests to determine whether he is fertile? **No / Yes,** the result was……

Chapter 4

Fertility preservation in female classic galactosemia patients

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ABSTRACT

Almost every female classic galactosemia patient develops primary ovarian insufficiency (POI) as a diet-independent complication of the disease. This is a major concern for patients and their parents, and physicians are often asked about possible options to preserve fertility. Unfortunately, there are no recommendations on fertility preservation in this group. The unique pathophysiology of classic galactosemia with a severely reduced follicle pool at an early age requires an adjusted approach. In this article recommendations for physicians based on current knowledge concerning galactosemia and fertility preservation are made. Fertility preservation is only likely to be successful in very young prepubertal patients. In this group, cryopreservation of ovarian tissue is currently the only available technique. However, this technique is not ready for clinical application, it is considered experimental and reduces the ovarian reserve. Fertility preservation at an early age also raises ethical questions that should be taken into account. In addition, spontaneous conception despite POI is well described in classic galactosemia. The uncertainty surrounding fertility preservation and the significant chance of spontaneous pregnancy warrant counseling towards conservative application of these techniques. We propose that fertility preservation should only be offered with appropriate institutional research ethics approval to classic galactosemia girls at a young prepubertal age.

INTRODUCTION

Almost every female patient with classic galactosemia, an inborn error of galactose metabolism, has primary ovarian insufficiency (POI) and is confronted with the struggle that reduced chances of having children presents (1). Patients and/or their parents often approach physicians with questions regarding fertility preservation. Answering these questions, however, remains difficult, due to the unique and yet to be determined underlying pathophysiological mechanisms of POI as well as the lack of experience with fertility preservation techniques in general and in girls with classic galactosemia in particular.

Classic galactosemia (ORPHA79239) is caused by deficient activity of galactose-1-phosphate uridyl transferase (GALT), as a result of mutations in the *GALT* gene located on chromosome 9p13. GALT is the second of the three enzymes in the Leloir pathway, the main pathway of galactose metabolism. The incidence of classic galactosemia varies between 1:16.000 (2) and 1:60.000 (3) in Western countries. Galactose is needed for energy metabolism and glycosylation of complex molecules. It may be derived from exogenous (dietary) sources, most importantly lactose from dairy products, or endogenous production. Deficiency of the GALT enzyme leads to accumulation of galactose and its metabolites and results in secondary glycosylation abnormalities. Patients usually present in the first weeks of life with signs of liver and renal disease, cataracts and an Escherichia coli sepsis. Diagnostic tests include elevated galactose and galactitol in body fluids, elevated Gal-1-P in erythrocytes, severely diminished enzyme activity in erythrocytes and mutations in the *GALT* gene. A galactose-restricted diet quickly resolves the early signs, but cannot prevent the development of later-onset complications, such as cognitive impairment, neurological sequelae, bone health abnormalities, and, in female patients, POI with subsequent infertility.

Although POI in classic galactosemia represents a major concern for these patients and/or their parents (4), there are no published recommendations concerning fertility preservation in this group. The current guidelines in patients with cancer (5-10) may be useful in assessing the risk-benefit ratio for girls with galactosemia. However, unlike cancer patients, the mechanisms causing ovarian dysfunction are unknown and the timing of onset may begin as early as prenatally (1, 11-14). Therefore, intervening when the patient reaches adulthood may be too late. As a consequence, applying fertility preservation options in very young girls raises medical, legal and ethical questions. Furthermore, spontaneous pregnancies do occur in this disease, demonstrating that conception is possible in some patients.

The aim of this paper is to discuss the different aspects of fertility loss in female classic galactosemia patients from a multidisciplinary perspective, and to propose recommendations for fertility preservation, taking into account pathophysiology, chances of spontaneous pregnancies and ethical issues. These recommendations are based on our experiences with classic galactosemia patients and the currently available literature.

Pathophysiology

Symptoms of POI differ between affected women, varying from subfertility, to early development of irregular menstrual cycles and infertility, to primary amenorrhea and absence of spontaneous puberty (15). The cause of POI in classic galactosemia is not yet understood. Several mechanisms have been postulated, including direct toxicity of metabolites (i.e. galactose-1-phosphate), altered gene expression, or aberrant function of hormones and or receptors due to glycosylation abnormalities (1, 16-18). It is also possible that not one, but several mechanisms act in unison to cause POI in classic galactosemia.

In general, POI can be caused by either the formation of a smaller primordial follicle pool or more rapid loss of primordial follicles (15) and there is evidence for both mechanisms in classic galactosemia. In classic galactosemia there is some evidence that the follicle pool at birth is as large as in girls without this disease. In two galactosemic neonates, morphologically normal ovaries with abundant oocytes and normal folliculogenesis have been reported (19, 20). In another patient, ultrasound showed apparently normal ovaries at age 7 (21). These studies suggest that the primordial follicle complement is normal initially, but comprehensive evidence is lacking.

Histological findings show that the ovaries contain a strongly reduced number of follicles at older, but variable ages. Histological findings in adolescents (aged 16 or 17 years) reveal a strongly reduced number of follicles, varying from far fewer follicles than expected for age (14) to almost complete absence (22-24). Data in younger adolescents or children are lacking. Histological findings in two adults showed absence of ovarian parenchyma (25) and an extremely reduced number of follicles in one subject (26), and a follicle number within the normal range in the other (26). Ovarian size and volume in patients aged 9-21 years assessed with MRI were comparable to those found in postmenopausal controls (27). Ultrasound or laparoscopy/laparotomy in patients during adolescence or adulthood often shows hypoplastic (24, 28, 29) or streak ovaries (22, 23, 25, 30), consistent with the absence of follicles. Of note, streak ovaries were also found during adolescence in the girl with apparently normal ovaries at age 7 (21). Taken together, it is possible that a normal complement of primordial follicles forms, but undergoes atresia more rapidly and that the ovaries can be severely damaged in girls at very young prepubertal age.

There is also evidence for defective follicle maturation, which may result from a paucity of follicles able to respond to stimulation or inability of the follicle to respond to gonadotropins in galactosemia. The few follicles present in the ovaries of classic galactosemia patients are mainly of the primordial type, and maturing follicles are rarely seen (14, 22, 23, 26, 28). Consistent with the absence of estrogen production due to absent ovarian activity (26, 27, 31), hypoplastic prepubertal uteri were observed in patients who did not receive estrogen supplementation (21-23, 29, 30).

Animal studies suggest that galactose might already have a direct toxic effect in fetal life. After feeding pregnant and lactating Sprague-Dawley rats a high galactose diet, the offspring had a significant and striking reduction in the number of small follicles, (11), a reduced number of primordial germ cells (PGCs) and a consequently smaller gonadal size (32) suggesting impaired germ cell migration, and a deficient complement of follicles in some of the exposed animals (33).

An accelerated loss of follicles during life has also been suggested in several studies. Lai et al found that the number of apoptotic granulosa cells of maturing follicles was higher in Sprague-Dawley rats fed a high galactose diet than in animals receiving normal chow (34). The same has been concluded in a recent study by Banerjee et al (35). Their results in isolated granulosa cells suggest that galactose exposure leads to an increased expression of p53, a protein mediating intrinsic death pathways in cells (36), consequently causing follicular atresia. The authors also suggest that attenuated FSH bioactivity is the underlying cause of the increased p53 expression. The latter is not consistent with failure of follicle response to exogenous FSH (27, 37).

Some animal studies also point to a compromised maturation. A high galactose diet significantly decreased the number of healthy growing and antral follicles, whereas the number of primordial or total atretic follicles was not affected (38), although other studies suggested that the primordial follicle count was lower (11). Similarly, other studies using the same model demonstrated a decreased ovulatory response (33, 39, 40), evidenced by reduced numbers of corpora lutea.

Both case reports of neonates compared to older girls and the animal studies suggest that galactosemia results in increased follicle apoptosis with an accelerated follicle loss of either primordial follicles or maturing follicles as the cause of POI (34, 35, 38). It might be very difficult to determine whether the large follicle pool present at birth, consisting of millions of primordial follicles, is reduced in classic galactosemia patients unless the decrease is marked. Animal models indicate a prenatal toxic effect of galactosemia but these animal studies, in which animals are fed a high galactose diet, might not represent human classic galactosemia completely. Although the histological and imaging data are very limited and call for a tedious conclusion, taken together, there seems to be a rapid decline in the number of primordial follicles either in fetal life or in early life in this disease, which has to be taken into account when discussing fertility preservation.

Spontaneous pregnancies in women with classic galactosemia

Over the past years it has become increasingly clear that spontaneous pregnancies in classic galactosemia occur, and occur more frequently than previously thought. In a cohort of 22 patients with classic galactosemia and POI, 9 patients tried to conceive, of which 4 succeeded (44 percent) (41). Although significant, the small sample size warrant further studies in a larger cohort. Many patients were told in the past that they had

no chance of conceiving and therefore may not have tried, which makes it difficult to determine the exact chance of spontaneous pregnancy in classic galactosemia. It is important that patients are aware of the occurrence of spontaneous pregnancies (2, 31, 41-43), allowing them both to try to conceive spontaneously and to avoid unplanned pregnancies. The World Health Organization defines infertility as a failure to achieve spontaneous pregnancy within twelve months or more (44). Therefore we advise physicians to encourage their patients to try to achieve spontaneous pregnancy for at least this period of one year. We also advise physicians to explain that pregnancy should not be postponed unnecessarily to increase chances of conception. Importantly, classic galactosemia women became pregnant despite postmenopausal FSH levels, extremely low estrogen concentrations, undetectable Müllerian Inhibitory Substance (MIS) or anti-Müllerian hormone levels (31, 45) and the severe Q188R/Q188R genotype (2, 41, 43). Therefore, genotype, enzyme activity in erythrocytes, and measurement of markers of ovarian reserve do not appear accurate predictors of pregnancy chances in this patient population. Besides, the ovaries of prepubertal girls are very small, even in healthy girls, limiting the relevance of imaging techniques in these patients. Of note, most of the women that became pregnant spontaneously have gone through normal puberty and reached menarche spontaneously, indicating that these might be predictive factors for an increased chance of spontaneous conception (41).

Fertility preservation in classic galactosemia patients

Importantly, fertility preservation data are mainly derived from cancer patients. The ovaries of a girl affected with non-ovarian cancer are healthy when there is a need for fertility preservation. In contrast, the ovaries of classic galactosemia girls are probably already damaged at an early age. Therefore, the success rate of the procedures is probably lower in galactosemic girls. Perhaps the most important issue to discuss before choosing to apply fertility preservation techniques is the fact that fertility preservation does not guarantee future pregnancy. A thorough evaluation including the abovementioned chance of spontaneous pregnancy and the risks and benefits needs to be taken into account. Three fertility preservation procedures currently offered to girls and women in need of fertility preservation are: a. ovarian tissue cryopreservation, b. mature oocyte cryopreservation and/or c. embryo cryopreservation. The latter two techniques require ovarian stimulation with FSH, whereas cryopreservation of ovarian tissue does not.

a. Ovarian tissue cryopreservation

In ovarian tissue cryopreservation, one ovary is harvested through laparoscopy and the cortex tissue is processed into cortical strips and frozen. When the patient is ready to conceive, the tissue is transplanted at either an orthotopic or a heterotopic location in the body. Currently, twenty healthy infants have been born worldwide as a result of

autotransplantation of the cryopreserved tissue (46). All mothers were women with no history of ovarian problems who were treated with chemotherapy after cancer diagnosis. However, the method is still experimental and consensus is lacking on the amount of tissue that needs to be harvested for cryopreservation (47, 48). The major risk of this technique is that the graft may not survive due to ischemic damage, which leads to massive follicular death (49, 50). As primordial follicles are more resistant to this damage (49), the chance of restoring fertility is related to the number and quality of the primordial follicles in the transplanted cortical tissue (51). Therefore, it is possible that the quantity of ovarian tissue removed should be influenced by the expected probability of POI (47). Another disadvantage of cryopreservation of ovarian tissue is that this technique might lower the chance of spontaneous pregnancy, as a result of reduction of the remaining ovarian pool. Moreover, it is also possible that the transplanted tissue is subject to the same detrimental factors that cause POI in this patient group.

b. Mature oocyte cryopreservation

In oocyte cryopreservation the patient's ovaries are stimulated with exogenous FSH, the growing follicles are punctured transvaginally and the oocytes are extracted from the follicles. The metaphase II oocytes are frozen using vitrification, a technique in which high initial concentrations of cryoprotectant and ultra-rapid cooling are used to create a glass-like state in a cell without forming ice crystals. Once the patient desires to conceive, the oocytes are warmed and consequently fertilized in a petri dish using a partner's or donor's semen (intracytoplasmatic sperm injection, ICSI). The procedure of mature oocyte cryopreservation is no longer considered experimental by multiple international reproduction societies, including the American Society for Reproductive Medicine (ASRM) (52). A recent review of the literature and meta-analysis suggested that fertilization and pregnancy rates were similar with vitrified/warmed oocytes and fresh oocytes (52). However, a limitation of this analysis is that the majority of data is derived from studies with oocytes obtained from healthy, young donors. The current recommendations by the ASRM state that oocyte cryopreservation is recommended for patients facing infertility due to chemotherapy or other gonadotoxic therapies, but that more widespread clinic-specific data on the safety and efficacy of oocyte cryopreservation in donor populations are needed before universal donor oocyte banking can be recommended. Therefore, its use in patients with galactosemia is as yet unclear.

c. Embryo cryopreservation

A third fertility preservation option is embryo cryopreservation, a well-established and successful technique. This procedure consists of ovarian stimulation, transvaginally harvesting cumulus oocyte complexes and extraction of oocytes. The oocytes are fertilized *in vitro* using a partner's or donor's semen. The fertilized oocytes also develop *in vitro* to the embryo stage and are then cryopreserved by means of vitrification or slow freezing. Once the patient desires to conceive embryos are warmed and implanted in

the uterus. The embryo survival rate is approximately 90% when vitrification is used (53). Pregnancy rates in regular IVF programs are on average 30% (54).

Since all fertility preservation techniques depend on the ovarian reserve (55), and considering the apparent rapid follicle pool decline in classic galactosemia, fertility preservation in these patients may need to take place during infancy or early childhood, seriously limiting the options. Techniques requiring ovarian stimulation are not suitable for prepubertal girls because of absence of maturation of the hypothalamic-hypophyseal-ovarian axis (56), leaving cryopreservation of ovarian tissue the only available technique for this age group. Fertility preservation in older, postpubertal classic galactosemia patients is likely to be unsuccessful as the ovarian reserve may be poor. Embryo cryopreservation and oocyte cryopreservation are, in theory, the best options in these patients. However, both techniques require ovarian stimulation. Ovarian stimulation in fifteen classic galactosemia patients, aged 15 to 36 years demonstrated poor estradiol production in all but one (27). Therefore, the severely reduced follicle pool limits the use of cryopreservation of embryos and mature oocytes (1). It might be an option for those young classic galactosemic women with a sufficient ovarian reserve, but these women probably are the women whose chances of spontaneous conception are highest.

If fertility preservation is not a reasonable option, or if pregnancy does not occur despite fertility preservation, another option is adoption or the use of donor oocytes to achieve pregnancy. The donor can be anonymous or can be a direct donor, such as a mother or sister. Mothers of galactosemic girls frequently propose to donate oocytes for their daughters. It may be necessary to cryopreserve the mother's oocytes for the future if the patient is not ready for children at the time of donation. The ethical issues resulting from intra familial donation are discussed below.

In the future, other fertility preservation techniques might become available. As an alternative for mature oocyte cryopreservation, cryopreservation of oocytes extracted from immature follicles has been evaluated (in vitro maturation, IVM). Another fertility preservation technique that is currently being evaluated and might be an option in the future is freezing and transplantation of the entire ovary including its vascular system (57, 58). Also the possible existence of oogonial stem cells (59) and induced pluripotent stem cells (60) might offer therapeutic options for the future.

Ethical considerations in fertility preservation

Ethical problems arise in all young patients in need of fertility preservation, regardless of the underlying etiology. Girls are often too young to decide about fertility preservation themselves and therefore their parents play a crucial role in this process. One probably cannot predict with certainty how she herself or, in case of the parents, their daughter will think about a pregnancy in the future. The parent's decision may not ultimately reflect the girl's wishes when she becomes an adult (8). In 2005, the American

Society for Reproductive Medicine stated that fertility preservation can be applied in minors if this is in the best interest of the child and if the girl gives her assent (6). If a girl is too young to give assent, parents may give consent if the procedure offers a net benefit for the child. Review of this decision by the ethics committee of a hospital or another independent body is in our opinion a must. The same criteria and advice hold true for fertility preservation techniques that are still considered experimental, though approved by the institutional ethics committee. Psychosocial guidance of the girl during the procedure and further discussion about fertility during adolescence and adulthood are very important. Moreover, legislation always plays a role in medical decision-making, but laws often differ between countries and may not be specific.

Cryopreservation of ovarian tissue, the most plausible fertility preservation option for this population, is still considered experimental and pregnancy rates resulting from this procedure are uncertain (9). Recommending methods that are not yet established may offer false hope (1). Therefore, the benefits and harms of every procedure should be discussed extensively, and chances of spontaneous pregnancy in this particular disease despite POI should be considered.

Intrafamilial oocyte donation might lead to ethical issues including psychosocial pressure on the donor and recipient, over-attachment of the donor to the offspring, and role confusion for the persons involved, including the resulting offspring (61). The process of intrafamilial donation requires the help of multiple professionals, a thorough screening of the donor, psychological counseling of both the donor and the recipient and extensive informing about legal parenting relations (61, 62).

DISCUSSION

As a consequence of the severe depletion of the follicle pool that probably occurs prenatally or early in childhood in classic galactosemia patients, fertility preservation in this group is only likely to be successful in very young patients. Oocyte cryopreservation after ovarian stimulation will likely not be successful in most women with classic galactosemia. It might be useful in women with a sufficient ovarian reserve who wish to postpone pregnancy. Ovarian tissue cryopreservation, the only available technique at a young age, has several important disadvantages, including the experimental character of the procedure and the reduced chances of spontaneous pregnancy. Therefore, the technique should probably not yet be offered in a clinical setting, but could be considered on research basis.

In general, classic galactosemia patients may have below-average cognitive functioning (2, 63-65) and social-emotional problems (66, 67), which might affect the ability to raise children. However, the severity of these impairments will not be clear at the time of fertility preservation, since this can change over time (68). At the time of fertility preservation, physicians should explain to the patient's parents that some patients

might not develop enough parental competency and that this might prevent the use of cryopreserved tissue. In adulthood, when the cognitive and ovarian phenotype of the patient is clear and when psychological tests have been performed, the adult patient can decide if she wishes to have children and if she wants to use the cryopreserved tissue in order to achieve this. At that time, physicians and the ethics committee of the hospital or another independent body should evaluate whether the patient has adequate parental competency, in case this is doubtful. This is consistent with other fertility preservation procedures, for instance in pediatric cancer patients (10).

Physicians confronted with questions regarding POI and fertility preservation in classic galactosemia, should emphasize that pregnancy has been repeatedly reported despite POI and they should not discourage patients to try to conceive spontaneously. Noteworthy, girls with a spontaneous puberty are likely to have higher chances of spontaneous pregnancy. Furthermore the risk of postponing pregnancy needs to be discussed as well, since in galactosemic women, as in any woman, fertility declines with age, lowering the risk of spontaneous conception. However, due to the unknown course of ovarian function and the unknown pathophysiological mechanism of POI in classic galactosemia, decisions about fertility and fertility preservation remain difficult for patients, parents and physicians.

CONCLUSIONS AND RECOMMENDATIONS

Based on the current knowledge about classic galactosemia and its pathophysiology, we recommend that fertility preservation should not be offered to these patients as common practice (Figure 1). If it is part of institutional research ethics approved research it might be taken into careful consideration. Cryopreservation of ovarian tissue in young prepubertal girls is at this moment the procedure of choice. At a later age, estimated success rates are too low since the ovarian damage is probably already too prominent. Trying to achieve spontaneous pregnancy seems to be the best option for older girls and women.

Another option for classic galactosemic girls and women might be oocyte donation, both anonymous donation and intrafamilial donation. We currently do not recommend intrafamilial donation by the mother, as mentioned above. If in a special situation oocyte donation by the patient's mother is considered, psychological screening and extensive counselling are required, according to previously mentioned guidelines (61).

Finally, we recommend engagement of an independent review board in the process of fertility preservation at minimum two points in the process: when the parental decision to preserve their daughter's ovarian tissue is considered, but also when the patient wishes to use the preserved tissue, as the parental capacity of the patient should be reviewed.

- Physicians should emphasize that spontaneous pregnancies occur in women with classic galactosemia, even after POI diagnosis.
- If fertility preservation is desired, cryopreservation at an early prepubertal age as a part of approved research currently seems to be the best option.
- The ethics committee of the hospital or another independent body should review the parent's decision before the fertility preservation procedure.
- The ethics committee of the hospital or another independent body should be involved in the decision making surrounding the use of the cryopreserved material.
- If a patient desires pregnancy, a one-year window for attempting spontaneous pregnancy is advised to avoid unnecessary use of assisted reproductive techniques.
- Anonymous or intrafamilial oocyte donation might be another option for classic galactosemia patients if pregnancy does not occur.

Figure 1: Our recommendations regarding fertility preservation in female classic galactosemia patients.

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Chapter 5

Exploration of the brain in rest: resting-state functional MRI abnormalities in patients with classic galactosemia

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ABSTRACT

Patients with classic galactosemia, a genetic metabolic disorder, encounter a variety of cognitive impairments, including motor (speech), language, and memory deficits. We used functional magnetic resonance imaging to evaluate spontaneous correlations in functional activity during rest to investigate potential abnormalities in neural networks. We characterized resting-state networks using seed-based correlation analysis in 13 adolescent patients and 13 matched controls. Patients showed alterations in networks encompassing the medial prefrontal cortex, pre- and postcentral gyrus, and inferior parietal lobule, involved in sensory-motor integration and spatial orientation. Furthermore, altered connectivity of networks including the insula, inferior frontal gyrus, pre-supplementary motor area, pre- and postcentral gyrus and superior parietal lobule - important for language production and motor (speech) planning - was demonstrated. Lastly, abnormalities were found in networks involving the occipital region, (pre)cuneus and lingual gyrus, possibly linked to visuospatial processing abnormalities. Importantly, across several seeds, altered functional connectivity to the lingual gyrus, precentral gyrus, insula and cuneus was observed in patients, suggesting special importance of these brain regions. Moreover, these alterations correlated with clinical test results, supporting a relation with the clinical phenotype. Our findings contribute to improved characterization of brain impairments in classic galactosemia and provide directions for further future investigations.

INTRODUCTION

The brain represents one of the target organs of damage in classic galactosemia, an inborn error of galactose metabolism, resulting in chronic impairments with significant impact on quality of life and general performance (1-3). Patients with classic galactosemia suffer from a profound deficiency of galactose-1-phosphate uridylyltransferase (GALT) due to mutations in the *GALT* gene, and affected newborns present with a severe toxicity syndrome upon exposure to galactose in milk. Immediate initiation of galactose-restriction is life-saving, but fails to prevent the development of burdensome brain impairments, including lower intelligence (4-8), language production and speech (motor) problems (9-13), slower information processing and memory deficits (4, 14), social difficulties (15), psychiatric conditions (16) and neurological sequelae (17).

The pathogenic basis of these debilitations is puzzling and there is an increasing number of studies providing evidence for structural changes in white and grey matter, as well as alterations at a functional level (17-28). Using innovative diffusion-weighted magnetic resonance imaging (MRI) analysis techniques, evidence for abnormal white matter microstructure in bilateral anterior tracts (reduced neurite density) and the left hemisphere (increased dispersion of neurites) was provided (27), which strengthens the hypothesis of aberrant myelin composition, possibly resulting from deficient galactocerebroside formation due to aberrant glycosylation (21, 25). Additionally, several studies demonstrated cerebral or cerebellar atrophy and decreased tissue density of grey matter (17-19, 21, 22, 24, 25, 28). This neuronal loss has been speculated to result from direct toxicity of galactose and its metabolites or aberrant glycosylation (25). In addition to reduced grey matter density, Timmers et al. (2016) also observed increased grey matter density in the inferior frontal and medial prefrontal cortex, which could reflect compensation for problematic motor and cognitive functions (memory, language) or abnormal maturation (28).

At a functional level, Dubroff et al. (2008) used [18F]fluorodeoxyglucose (FDG) positron emission tomography (PET) to evaluate glucose metabolism in patients (20). Decreased metabolism was found in the occipital region, orbital frontal lobes, sensorimotor areas, cerebellum, superior temporal lobes, and the mid and superior parietal regions. Increased metabolism was demonstrated in bilateral anterior cingulate cortex (ACC) and temporal poles, as well as in basal ganglia. The findings in ACC and basal ganglia were interpreted as showing potential compensation for problematic motor functions.

Recently, a task-based functional MRI (fMRI) study by our group, wherein patients carried out a language production task, was the first to point towards abnormal language-related brain networks in this disorder (26). Evaluation of neural activity demonstrated that patients recruited different and more extensive brain regions during language production as compared to controls. The left inferior frontal gyrus (IFG), the right insula and the left pre-supplementary motor area (pre-SMA) were subsequently select-

ed as seeds for a seed-based functional connectivity analysis, which revealed variations in functional connectivity in comparison to controls.

Resting-state fMRI studies, in which neuronal connectivity in absence of a specific stimulus or task is assessed, are increasingly conducted within multiple fields of neuroscience to study the organization of core processing systems of the brain. The rationale behind these studies is that the brain is always active and shows spontaneous neuronal activity even during rest. Brain regions that show synchronous neuronal activity are considered to be functionally connected, thereby constituting a functional network (29-32). A commonly used approach to study functional connectivity is the seed-based correlation analysis (SCA). This method is hypothesis-driven and correlates the resting-state time courses of an *a priori* selected region of interest (ROI; i.e. seed) to the time series of the rest of the brain (29, 31, 33, 34), wherein the seed is selected from a task-dependent activation map or anatomically based on previous literature (35). Despite differences in data acquisition and analysis methods, multiple resting-state networks have been consistently found across functional connectivity studies (30, 36, 37). Additionally, altered functioning of these networks has been demonstrated in participants suffering from a variety of brain disorders (38), including neurodegenerative diseases such as Alzheimer's disease (39-41), as well as attention deficit hyperactivity disorder (ADHD) (42-44) and other psychiatric conditions.

In our previous study, we focused on functional networks that are involved in an active task (26). However, resting-state fMRI allows us to investigate the overall functional organization of brain networks, which has not been studied before in classic galactosemia. The current study therefore characterized functional networks in resting-state in patients with classic galactosemia as compared to healthy controls using a seed-based approach. Seeds were selected based on previous neuroimaging studies in this population, namely the left IFG, right insula, left pre-SMA, right and left putamen, right and left medial prefrontal cortex (mPFC), and the medial occipital region (20, 26, 28). Findings were correlated to available behavioral outcome measures as well, in order to confirm potential relations between functional networks and behavior (45). For this, measures of functional connectivity of brain regions of interest were correlated to neurocognitive test results. Our aim was to elucidate affected brain networks in patients with this disease to improve the characterization of brain impairments encountered by these patients.

MATERIAL AND METHODS

Participants

Thirteen adolescent patients with classic galactosemia and thirteen age- and gender-matched healthy controls participated in this study. Participants were recruited as de-

scribed by Timmers et al. (2015) (26). Briefly, patients who participated in an earlier study of our group and aged 14 years or older were invited to participate in the current study. Classic galactosemia diagnosis was confirmed by GALT enzyme activity measurement and/or *GALT* mutation analysis. One patient and one control subject were excluded because of extensive motion during scanning and a current health condition, respectively. Characteristics of included patients and controls are presented in Table 1. Participants had no other relevant health conditions, had normal or corrected to normal vision, were native Dutch speakers, and were eligible for MRI assessment. All gave written informed consent, and in case of minors informed consent was obtained from the parents/caregivers as well. The Medical Ethical Committee of the University Hospital Maastricht/University of Maastricht (azM/UM) gave ethical approval for this study. The study was conducted in accordance with the Declaration of Helsinki.

Table 1: Participant characteristics for both patient and control group.

	Patients (n=12) Value (range)	Controls (n=12) Value (range)
Gender	Males: 3 Females: 9	Males: 3 Females: 9
Age (years) [1]	17.4 (14.6 - 21.1)	17.1 (14.0 - 20.0)
Age at initiation of galactose-restricted diet (days)	11.0 (0 - 35)	
GALT activity (% of reference value) [2]	0.55 (0 - 1.52)	
GALT mutation	5x p.Q188R/p.Q188R (42%) 1x p.Q188R/p.L195P (8%) 3x p.L195P/p.K229N (25%) 2x p.W134fs/unknown (17%) 1x Unknown (8%)	
Special education (% of group) [3]	75	
Speech therapy (% of group) [3]	92	
Motor therapy (% of group) [3]	42	
Visuospatial working memory [4]	30 (<20-51)	
Verbal working memory [5]	4.25 (2-7)	
Sustained attention [6]	14.9 (11.3-20.3)	

1: Median age was not significantly different between groups ($F_{1,22} = 0.12$, $p = 0.73$)
2: GALT enzyme activity measured at diagnosis
3: Special education, speech therapy or motor therapy at some point in life
4: Immediate Recall subtest of Rey Osterreith Complex Figure T-score (Meyers and Meyers 1995)
5: Digit Span (Forward and Backward) score (Van Haasen et al. 1986)
6: Bourdon-Vos mean reaction time (Vos 1988)

Procedure

To prevent excessive motion during scanning, participants practiced lying in a dummy scanner. After this practice session, participants received explicit instructions and were placed comfortably in the MRI scanner. Resting-state data were acquired, followed by a language task. The task-evoked functional connectivity pattern analyses were described elsewhere (26). During the resting-state scan (duration: 6 minutes) a fixation point was presented and participants were instructed to relax, not to think of something in particular and to keep their eyes open. Total duration of the experiment, including the acquisition of other data, was approximately 90 minutes. Afterwards, participants completed a short questionnaire addressing the difficulty of the session (not significantly different between patients and controls; $t = 0.562$, $df = 24$, $p = 0.58$).

Data acquisition

Data were obtained on a 3T Siemens MAGNETOM® Allegra head scanner using an 8-channel head coil, and a 3T Siemens MAGNETOM® Trio whole body scanner using a 32-channel head coil (Siemens Medical System, Erlangen, Germany). Data acquisition on two different scanners was necessary as a result of irresolvable technical issues with the Allegra scanner. Four patients and four controls were scanned on this MRI scanner. Parameters of the Allegra scanner and Trio scanner were identical unless otherwise specified.

T1-weighted anatomical images were acquired using an ADNI MPRAGE sequence with 192 slices and 1mm iso-voxel resolution covering the whole brain (repetition time [TR] = 2250 ms; echo time [TE] = 2.6 ms). Functional T2*-weighted resting-state images were obtained using a standard echo-planar imaging (EPI) sequence covering the whole brain, except for cerebellum (TR = 2000 ms, TE = 30 ms, 32 slices, 180 volumes, 3.5 mm iso-voxel).

Data pre-processing

Data were analyzed using BrainVoyager QX version 2.8.4.2645 (Brain Innovation, Maastricht, the Netherlands). For functional datasets, the first four volumes of each complete time series were discarded because of saturation effects. Pre-processing of the functional data included correction for slice time differences, 3D head motion correction (none of the six parameters differed significantly between patients and controls, $p > 0.05$ for all parameters), linear trend removal and spatial smoothing (Gaussian filter FWHM of 4 mm). Functional datasets were co-registered with anatomical data and normalized in Talairach space (3.5 mm iso-voxel). Anatomical data of all participants were averaged to create a group-based dataset.

Statistical analyses

We conducted a SCA with the left IFG, right insula, left pre-SMA, right and left putamen, right and left mPFC, and the medial occipital region as seeds (a sphere was created surrounding the peak voxel and taken as region of interest [ROI], 257 voxels; Table 2). The six detrended 3D head motion parameters and their derivatives, head motion spikes (not significantly different between patients and controls, t = -0.974, df = 22, p = 0.34), the extracted mean signal from the cerebral spinal fluid (CSF) and white matter (WM), and the global signal were Z-normalized and added as variables of no interest. Though the approach remains a subject of debate (73, 74), we decided to regress out the global signal since we are interested in localized network-specific neuronal activity, which could be obscured by unspecific global blood oxygenation level dependent (BOLD) fluctuations. Furthermore, signal oscillations at a frequency of 0.1 – 0.25 Hz (sine-cosine pairs) were added as confounders for low-pass filtering of the time series.

The BOLD time courses of the seeds were extracted, normalized and correlated with the time series from all other brain voxels using a random-effects (RFX) group analysis. We first estimated functional connectivity for each participant separately in a multi-study RFX General Linear Model analysis (first level), which then served as input for a second-level analysis of variance (ANOVA), allowing group contrasts. Functional connectivity per group was inspected at the level of the whole cerebrum on maps corrected at q(FDR) < 0.05. Statistical contrasts between patients and controls were inspected at the level of the whole cerebrum on maps corrected at a statistical threshold of p < 0.005. The significant group differences were converted to volumes of interest (VOIs) with a cluster threshold of 150.

Table 2: Regions of interest (ROIs) used as seeds in seed-based correlation analysis

	Mean X Talairach coordinate	Mean Y Talairach coordinate	Mean Z Talairach coordinate
Left IFG	-49	13	22
Left pre-SMA	-6	-2	62
Right insula	32	14	6
Right mPFC	3	45	34
Left mPFC	-8	48	28
Right putamen	21	15	1
Left putamen	-17	9	4
Occipital region	2	-81	2

Mean Talairach coordinates (center of gravity) for seeds used in seed-based correlation analysis are presented. Abbreviations: IFG = inferior frontal gyrus, pre-SMA = pre-supplementary motor area, mPFC = medial prefrontal cortex.

Correlations with neurocognitive tests

From clusters in which group differences were observed, beta values were extracted. In the patient group, the beta values per cluster (representing the functional connectivity of that region with the seed region) were correlated with results of neurocognitive tests using a Pearson correlation analysis. The Immediate Recall subtest of the Rey Osterreith Complex Figure (expressed as T-score) was used to evaluate visual working memory (75). The Digit Span (Forward and Backward) measured verbal working memory (76). The Bourdon-Vos test was used to assess sustained attention (mean reaction time [RT]) (77). Correlations with a p value < 0.05 were considered statistically significant (no multiple comparison correction was performed due to the limited sample size, but only strong correlations > |0.6| were reported (78)).

RESULTS

Group differences in seed-based correlation analysis

Medial prefrontal cortex

In controls, activity in the right mPFC seed region correlated with left and right middle temporal gyrus (MTG) and the bilateral posterior cingulate cortex (PCC) and precuneus (Pc) (Figure 1A). Furthermore, the bilateral ACC, left IFG and bilateral inferior temporal gyrus were correlated. In patients a generally similar, yet more extensive network was revealed on a q(FDR) corrected map (Figure 1A). More extensive regions of the frontal lobe, right precuneus, PCC, right MTG and ACC were correlated with the right medial frontal seed, and in addition the right parietal region was correlated. Statistical comparison between the two groups confirmed increased connectivity with the right superior frontal gyrus (SFG), right IFG, right inferior parietal lobule (IPL), right PCC, left mid cingulate cortex (MCC), right precuneus and right MTG in patients, as well as with right lingual gyrus, left cuneus and left insula (Figure 1A, Table 3). Decreased connectivity was demonstrated in left medial frontal gyrus (MeFG), right parahippocampal gyrus, bilateral precentral gyrus (PrG), left postcentral gyrus (PoG), left pre-SMA, left IFG, left precuneus and left MTG.

Connectivity maps showed that the left mPFC seed was functionally connected with the surrounding medial and superior frontal area, as well as with the dorsal ACC (bilaterally) in the control group (Figure 1B). In patients, activity of the seed correlated with more extensive parts of the left and right frontal regions, as well as with the left middle temporal lobe and both parietal lobes (right > left) (Figure 1B). Statistical tests corroborated increased functional connectivity in patients between the seed and bilateral SFG, left MeFG, left IPL, left MTG, left superior temporal gyrus (STG), right ACC and left PCC (Figure 1B, Table 3). Decreased functional connectivity was found with right MeFG, left

MTG, bilateral PoG, right PrG, bilateral IFG, right IPL, right STG, bilateral (pre)cuneus and left insula.

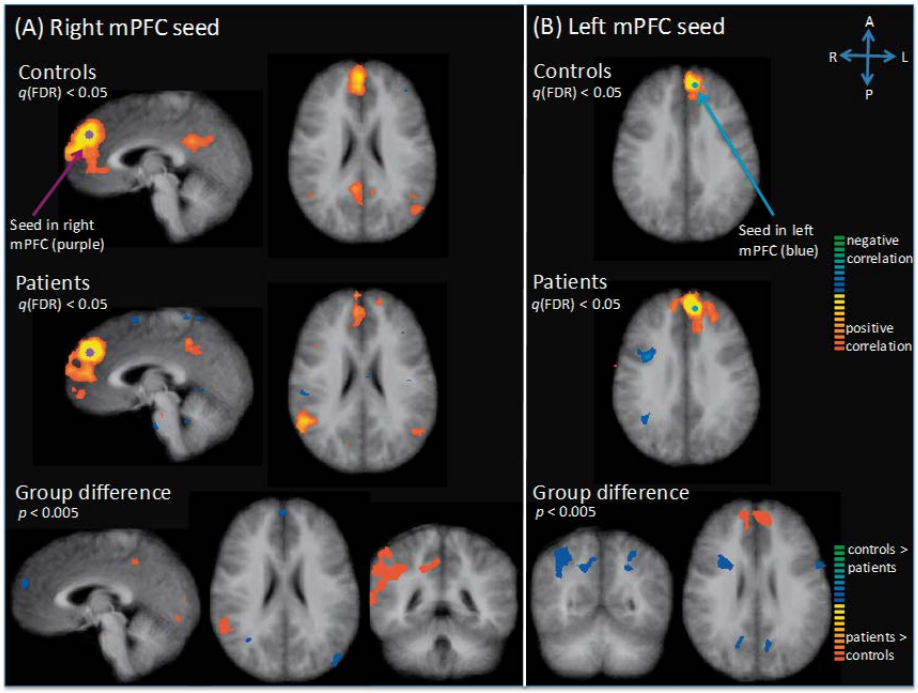

Figure 1: Functional connectivity (Fc) maps for the medial prefrontal cortex (mPFC) as seed; panel a: right mPFC seed, panel b: left mPFC seed.
Upper and middle row: Statistical maps resulting from the seed-based correlation analysis separate per group (red indicates positive correlation, blue indicates negative correlation). Seeds are overlaid in purple (right mPFC seed) or blue (left mPFC seed). Maps are thresholded at $q(FDR) < 0.05$. Lower row: Group differences in functional connectivity per seed region (red indicates patients show increased connectivity as compared to controls, blue indicates patients show decreased connectivity as compared to controls). Maps are corrected at $p < 0.005$ with a cluster threshold of 150.

Putamen

The seed in the right putamen showed temporal correlation with the head of the right nucleus caudatus and right thalamus, as well as with a small region of the right IFG (Figure 2A). In the patient group, a more extensive region of the right IFG was correlated. Furthermore, additional correlations were seen with right insula, right ACC and right MCC (Figure 2A). Statistical comparisons between patients and controls demonstrated increased functional connectivity with right PoG, right IFG, left superior parietal lobule (SPL) and left temporal lobe in the patient group (Figure 2A, Table 3). Decreased func-

tional connectivity was found in right MTG, right thalamus, bilateral PCC and left cerebellum.

In controls, activity in the left putamen seed correlated with activity in the left nucleus caudatus, left thalamus, right nucleus caudatus and right putamen (Figure 2B). A similar network was shown in patients. However, additional correlations with the left ACC and right thalamus were seen on the *q*(FDR) corrected map (Figure 2B). The statistical comparison revealed decreased connectivity with left SPL and left lingual gyrus in patients (Figure 2B, Table 3).

Table 3: Group differences per seed and significant correlations with neurocognitive tests

	Group difference	Nr of voxels	Peak Talairach coordinate			Significant correlation (patients only)
			X	Y	Z	
Lef IFG – Cl. 1	c>p	300	9	26	22	
Left IFG – Cl. 2	p>c	170	-9	11	52	
Left IFG – Cl. 3	c>p	164	-39	-61	49	
Right Insula – Cl. 1	c>p	227	48	-4	10	Verbal working memory (r = 0.60; p = 0.04)
Right Insula – Cl. 2	p>c	241	33	17	7	
Right Insula – Cl. 3	p>c	921	24	50	16	
Right Insula – Cl. 4	p>c	305	-6	8	49	
Right Insula – Cl. 5	c>p	168	-30	-16	34	
Right Insula – Cl. 6	c>p	158	-60	-19	19	
Left pre-SMA – Cl. 1	p>c	182	-21	-52	70	
Occipital region – Cl. 1	c>p	182	22	-88	22	
Occipital region – Cl. 2	c>p	2460	-12	-97	7	Verbal working memory (r = -0.60; p = 0.04)
Occipital region – Cl. 3	c>p	412	-33	-73	-11	Sustained attention (r = -0.60; p = 0.04)
Occipital region – Cl. 4	c>p	196	-42	-13	55	Verbal working memory (r = -0.62; p = 0.03)
Left putamen – Cl. 1	c>p	448	-22	-67	61	
Left putamen – Cl. 2	c>p	195	-30	-82	-5	Visuospatial working memory (r = -0.62; p = 0.03)
Right putamen – Cl. 1	c>p	445	51	5	-14	
Right putamen – Cl. 2	p>c	187	51	-22	43	
Right putamen – Cl. 3	c>p	211	51	-13	-14	
Right putamen – Cl. 4	c>p	2167	12	-25	10	
Right putamen – Cl. 5	p>c	724	21	17	-2	
Right putamen – Cl. 6	c>p	995	-9	-37	7	
Right putamen – Cl. 7	c>p	150	-3	-52	-14	
Right putamen – Cl. 8	p>c	192	-24	-49	46	
Right putamen – Cl. 9	p>c	246	-39	-49	-2	

	Group difference	Nr of voxels	Peak Talairach coordinate			Significant correlation (patients only)
			X	Y	Z	
Left mPFC – Cl. 1	p>c	173	63	-7	22	
Left mPFC – Cl. 2	p>c	1040	67	14	10	
Left mPFC – Cl. 3	c>p	257	57	-40	19	
Left mPFC – Cl. 4	c>p	169	57	-31	13	
Left mPFC – Cl. 5	c>p	162	54	5	34	
Left mPFC – Cl. 6	c>p	857	45	41	4	
Left mPFC – Cl. 7	c>p	285	39	-28	65	
Left mPFC – Cl. 8	c>p	1509	33	5	31	
Left mPFC – Cl. 9	c>p	3119	30	-46	46	
Left mPFC – Cl. 10	c>p	160	39	2	-14	
Left mPFC – Cl. 11	c>p	344	36	-31	34	
Left mPFC – Cl. 12	p>c	208	30	38	4	
Left mPFC – Cl. 13	c>p	1876	18	-52	40	
Left mPFC – Cl. 14	c>p	247	18	50	4	
Left mPFC – Cl. 15	p>c	1232	15	32	7	
Left mPFC – Cl. 16	c>p	224	15	-64	7	
Left mPFC – Cl. 17	p>c	2724	-3	50	28	
Left mPFC – Cl. 18	c>p	444	12	2	19	
Left mPFC – Cl. 19	c>p	579	-3	-91	13	
Left mPFC – Cl. 20	p>c	224	-9	41	43	
Left mPFC – Cl. 21	c>p	333	-9	-64	28	
Left mPFC – Cl. 22	p>c	246	-12	47	1	
Left mPFC – Cl. 23	p>c	585	-21	44	22	
Left mPFC – Cl. 24	p>c	307	-15	-40	19	
Left mPFC – Cl. 25	p>c	320	-27	20	49	
Left mPFC – Cl. 26	c>p	336	-24	-55	37	
Left mPFC – Cl. 27	c>p	158	-27	53	4	
Left mPFC – Cl. 28	c>p	301	-27	-49	49	
Left mPFC – Cl. 29	c>p	550	-27	23	4	Visuospatial working memory (r = -0.67; p = 0.02
Left mPFC – Cl. 30	c>p	196	-36	-34	65	
Left mPFC – Cl. 31	c>p	258	-42	-7	-8	
Left mPFC – Cl. 32	p>c	188	-46	-67	46	
Left mPFC – Cl. 33	p>c	317	-51	18	-20	
Left mPFC – Cl. 34	p>c	308	-54	-64	19	
Left mPFC – Cl. 35	p>c	213	-57	-1	-17	
Left mPFC – Cl. 36	c>p	337	-54	8	31	

	Group difference	Nr of voxels	Peak Talairach coordinate			Significant correlation (patients only)
			X	Y	Z	
Right mPFC – Cl. 1	c>p	284	67	17	13	
Right mPFC – Cl. 2	p>c	8285	39	-40	37	
Right mPFC – Cl. 3	p>c	712	39	-7	-26	
Right mPFC – Cl. 4	c>p	208	45	8	37	
Right mPFC – Cl. 5	p>c	284	42	38	7	
Right mPFC – Cl. 6	c>p	1459	24	-67	59	
Right mPFC – Cl. 7	c>p	209	27	-31	-14	
Right mPFC – Cl. 8	p>c	506	15	-43	37	
Right mPFC – Cl. 9	p>c	211	15	20	49	
Right mPFC – Cl. 10	p>c	465	9	-88	-8	
Right mPFC – Cl. 11	p>c	309	-3	-91	13	
Right mPFC – Cl. 12	c>p	193	3	56	22	
Right mPFC – Cl. 13	p>c	188	0	-19	-20	
Right mPFC – Cl. 14	p>c	283	-6	-76	16	
Right mPFC – Cl. 15	c>p	209	-12	57	1	
Right mPFC – Cl. 16	c>p	189	-12	26	58	
Right mPFC – Cl. 17	c>p	313	-18	-52	52	
Right mPFC – Cl. 18	p>c	163	-15	-10	31	
Right mPFC – Cl. 19	p>c	232	-24	8	31	
Right mPFC – Cl. 20	p>c	449	-33	29	7	Visuospatial working memory (r = 0.70; p = 0.01)
Right mPFC – Cl. 21	c>p	167	-36	-7	49	Visuospatial working memory (r = -0.61; p = 0.04)
Right mPFC – Cl. 22	c>p	454	-48	5	-26	
Right mPFC – Cl. 23	c>p	306	-45	-22	43	
Right mPFC – Cl. 24	c>p	326	-48	-73	28	
Right mPFC – Cl. 25	c>p	317	-48	23	16	
Right mPFC – Cl. 26	c>p	511	-60	-7	-5	

Clusters (cl.) of difference in functional connectivity between controls (c) and patients (p) specified per seed (cluster threshold 150). Size and peak Talairach coordinates of each cluster are presented. Furthermore, significant correlations between clusters and neurocognitive test results are shown (for patient group only). Tests used: Rey Immediate T-score for visuospatial working memory (Meyers and Meyers 1995), Digit span for verbal working memory (Van Haasen et al. 1986), Bourdon-Vos mean Reaction Time for sustained attention (Vos 1988).

Left inferior frontal gyrus

The seed in the left IFG showed temporal correlation with the left MeFG, left PrG, bilateral MTG, right IFG, left IPL and SPL, left supramarginal gyrus, left precuneus and left

angular gyrus in controls (Figure 3A). The *q*(FDR) corrected map revealed a less extensive network in patients, with less extensive correlation between the seed and its surrounding area, the contralateral side, and the left parietal lobule (Figure 3A). Statistical tests indicated that functional connectivity in patients is increased in the left pre-SMA and decreased in right ACC and left SPL, as compared to controls (Figure 3A, Table 3).

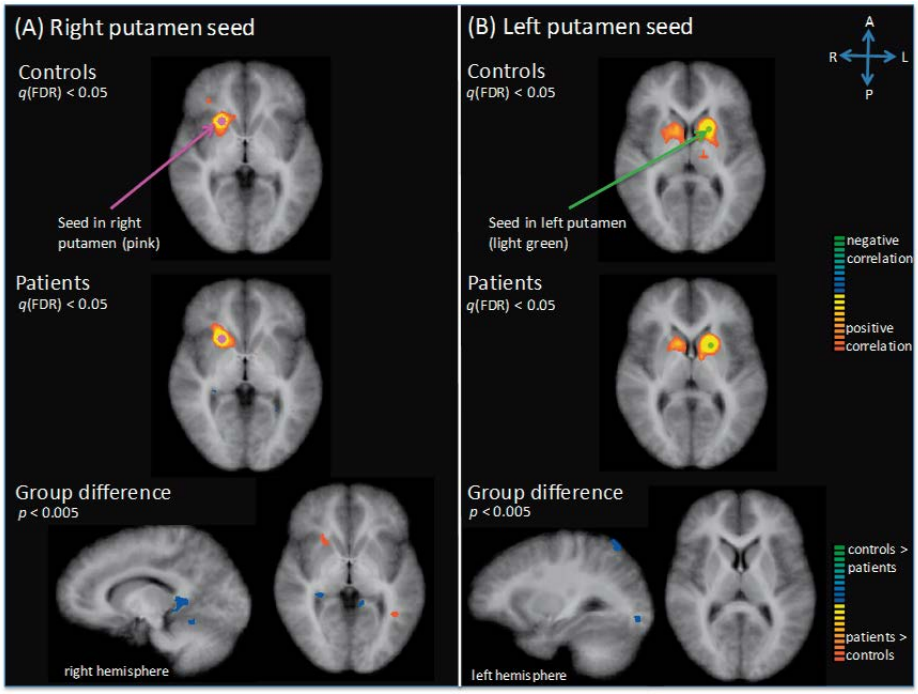

Figure 2: Functional connectivity (Fc) maps for the putamen seed; panel a: right putamen seed, panel b: left putamen seed.
Upper and middle row: Statistical maps resulting from the seed-based correlation analysis separate per group. Seeds are overlaid in pink (right putamen seed) or light green (left putamen seed). Maps are thresholded at *q*(FDR) < 0.05. Lower row: Group differences in functional connectivity per seed region (red indicates patients show increased connectivity as compared to controls, blue indicates patients show decreased connectivity as compared to controls). Maps are corrected at $p < 0.005$ with a cluster threshold of 150.

Right insula

In controls, activity in the right insula seed showed correlation with activity in right pre-SMA, bilateral PrG, bilateral PoG, bilateral ACC/MCC, right MFG, bilateral IFG, right nucleus caudatus, right putamen, right thalamus, right IPL, left insula and left cuneus (Figure 3B). More extensive correlation with bilateral pre-SMA regions and the right MeFG was noticed in patients, as compared with controls (Figure 3B). Less extensive correlation was seen with right basal ganglia and thalamus, left insula, left IFG and left PoG. The

statistical comparison corroborated increased connectivity with right anterior insula, right SFG and left pre-SMA in patients, as compared to controls, as well as a decreased connectivity with right posterior insula, right PrG and left PoG (Figure 3B, Table 3).

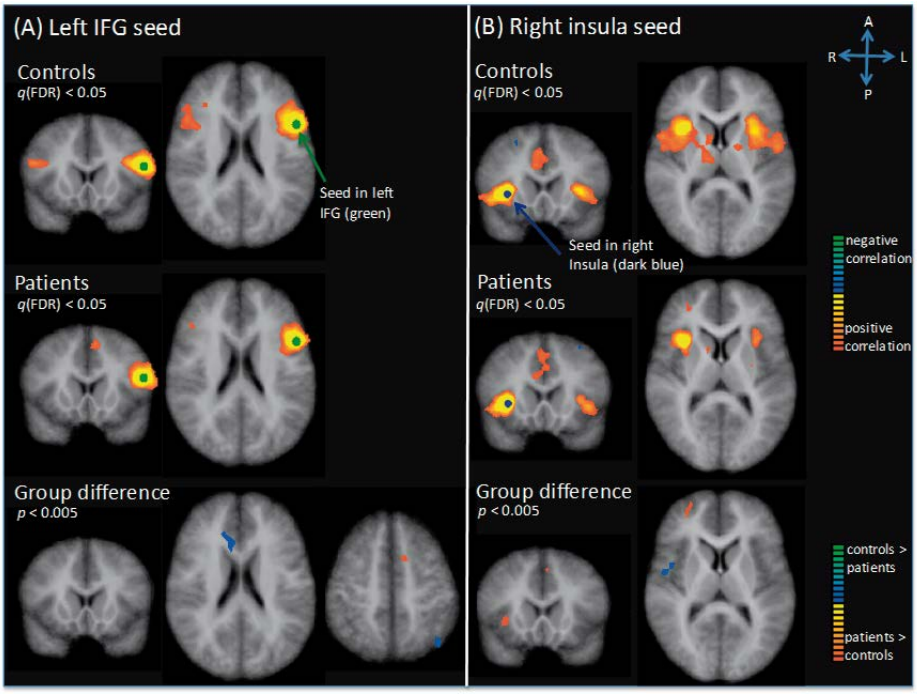

Figure 3: Functional connectivity (Fc) maps for the left inferior frontal gyrus (IFG) seed (panel a) and right insula seed (panel b).
Upper and middle row: Statistical maps resulting from the seed-based correlation analysis separate per group. Seeds are overlaid in green (left IFG seed) or dark blue (right insula seed). Maps are thresholded at q(FDR) < 0.05. Lower row: Group differences in functional connectivity per seed region (red indicates patients show increased connectivity as compared to controls, blue indicates patients show decreased connectivity as compared to controls). Maps are corrected at $p < 0.005$ with a cluster threshold of 150.

Left pre-supplementary motor area

Connectivity maps showed that the left pre-SMA seed was functionally connected with bilateral (pre-)SMA and premotor cortices and the left PrG in the control group (Figure 4A). A similar network was seen in patients. However, several additional regions were correlated, including the right PrG, the left MeFG and left SFG (Figure 4A). Statistical comparison showed that patients have increased connectivity between the seed and left SPL, as compared to controls (Figure 4A, Table 3).

Occipital region

Activity in the medial occipital region seed correlated with activity in bilateral middle occipital gyrus, bilateral lingual gyrus, extrastriate cortex, bilateral cuneus, the right precuneus, the right and left PrG in controls (Figure 4B). In the control group bilateral PrG, right pre-SMA and cerebellum were also correlated. In patients, less extensive correlation was seen with the occipital region surrounding the seed, especially bilateral middle occipital gyri, as well as with right precuneus, cerebellum and motor areas (Figure 4B). Statistical tests confirmed decreased functional connectivity in patients with regard to bilateral cuneus, left lingual gyrus and left PrG (Figure 4B, Table 3).

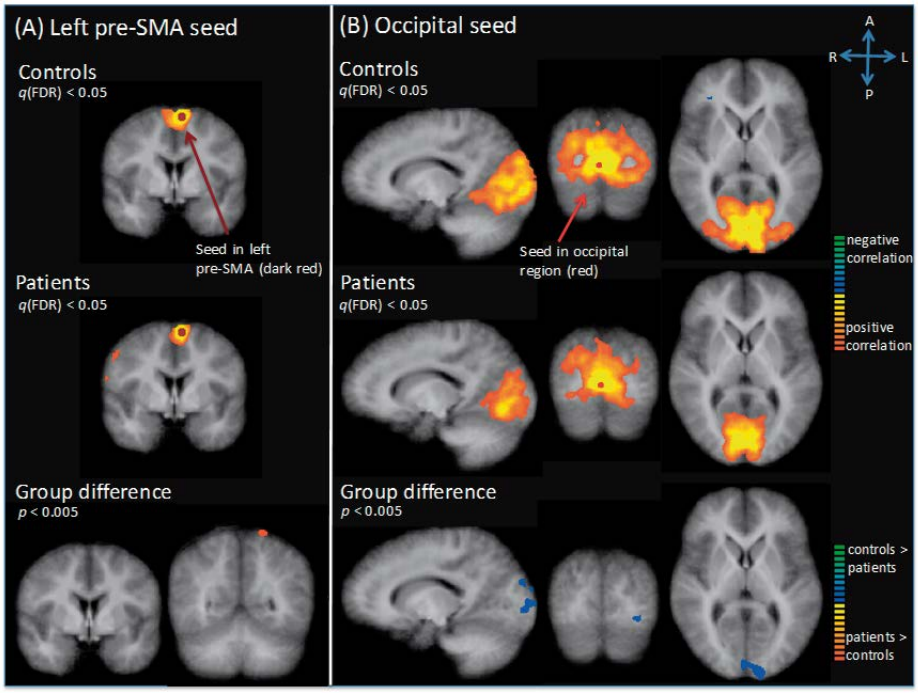

Figure 4: Functional connectivity (Fc) maps for left pre-supplementary motor area (SMA) seed (panel a) and occipital seed (panel b).
Upper and middle row: Statistical maps resulting from the seed-based correlation analysis separate per group. Seeds are overlaid in dark red (left pre-SMA seed) or red (occipital seed). Maps are thresholded at q(FDR) < 0.05. Lower row: Group differences in functional connectivity per seed region (red indicates patients show increased connectivity as compared to controls, blue indicates patients show decreased connectivity as compared to controls). Maps are corrected at $p < 0.005$ with a cluster threshold of 150.

Correlations with neurocognitive tests

A significant correlation between the patients' beta values (representing the functional connectivity with the seed region) and results of neurocognitive tests was found for eight clusters (Table 3, Figure 5 (A)). Functional connectivity between the left putamen and the left lingual gyrus (cluster 2, which showed decreased functional connectivity in patients, as compared to controls) was negatively correlated with visuospatial working memory (r = -0.62, p = 0.03), i.e. increased connectivity was correlated with lower performance in patients. The same holds true for connectivity between the right mPFC and left PrG (cluster 21, which showed decreased functional connectivity in patients, as compared to controls) (r = -0.61, p = 0.04), i.e. increased connectivity was correlated with lower performance on visuospatial working memory in patients. Correlations of functional connectivity patterns with the left anterior insula (left mPFC cluster 29 and right mPFC cluster 20) were two-sided. Functional connectivity between the left mPFC and left anterior insula (cluster 29, which showed decreased functional connectivity in patients, as compared to controls) was negatively correlated with visuospatial working memory (r = -0.67, p = 0.02) (i.e. increased connectivity was correlated with lower performance in patients), whereas functional connectivity between the right mPFC and the left anterior insula (cluster 20, which showed increased functional connectivity in patients, as compared to controls) was positively correlated with visuospatial working memory (r = 0.70, p = 0.01) (i.e. increased connectivity was correlated with higher performance in the patient group).

Functional connectivity between the right insula seed and the right posterior insula (cluster 1, which demonstrated decreased functional connectivity in patients, as compared to controls) was positively correlated to verbal working memory (r = 0.60, p = 0.04), i.e. increased connectivity was correlated with higher performance in the patient group. Functional connectivity between the occipital region and left cuneus (cluster 2, which showed decreased connectivity in patients, as compared to controls), and between the occipital region and left PrG (cluster 4, which also showed decreased connectivity in patients) as well, were both negatively correlated with verbal working memory (r = -0.61, p = 0.04 and r = -0.62, p = 0.03, respectively), i.e. higher functional connectivity was correlated with lower performance in patients.

Finally, functional connectivity between the occipital region and left lingual gyrus (cluster 3, which showed decreased connectivity in patients, as compared to controls) was negatively correlated to sustained attention (r = -0.60, p = 0.04), i.e. increased connectivity was correlated with lower performance in the patient group.

DISCUSSION

In the current study, we explored intrinsic functional networks by investigating spontaneous correlations in functional activity during rest in patients with classic galactosemia. The right and left mPFC, right and left putamen, left IFG, right insula, left pre-SMA and occipital region were selected as seeds for a SCA, based on previous studies indicating altered structure, function and/or metabolic activity of these brain regions (20, 26, 28). Results from the SCA point towards several differences in these resting-state functional networks between patients with classic galactosemia and age- and gender-matched controls. Importantly, group differences per seed show substantial overlap. Independent of the seed region, differences in connectivity across groups were observed within the IPL and SPL, precuneus, cuneus, lingual gyrus, insula, IFG, PrG and PoG. These results indicate a special role of these brain regions within the altered brain connectivity in this patient group (Figure 5).

The right and left mPFC seed analysis indicated decreased connectivity with the PrG and PoG, and increased connectivity with the IPL. Assuming that decreased connectivity relates to impaired cognition and behavior, impaired connectivity of prefrontal executive functions and sensory-motor integration may relate to problems in the patients motor sequence planning, including motor speech production and planning difficulties (46, 47). This would be in agreement with previously described motor (speech) disorders in classic galactosemia (9, 11, 17, 48). Assuming that increased connectivity relates to compensatory functions, the observed increased connectivity of mPFC and IPL could be related to elevation of attention. Enhanced attention is needed to conduct a more difficult task (49), and the IPL is a relevant region in this attention network (50, 51). Increased connectivity might also relate to compensation of motor sequencing problems via recruitment of spatial information, as IPL has a relevant role in spatial orientation (52).

For both frontal seed regions, alterations in connectivity with the precuneus and cuneus were seen, which might be related to visuospatial and working memory deficits as these areas have been reported involved in these tasks (53-55). This interpretation is in line with the below average scores on visuomotor/-perceptual/-spatial functioning tests and working memory assessments found in patients with classic galactosemia (4, 5, 7, 14, 56, 57).

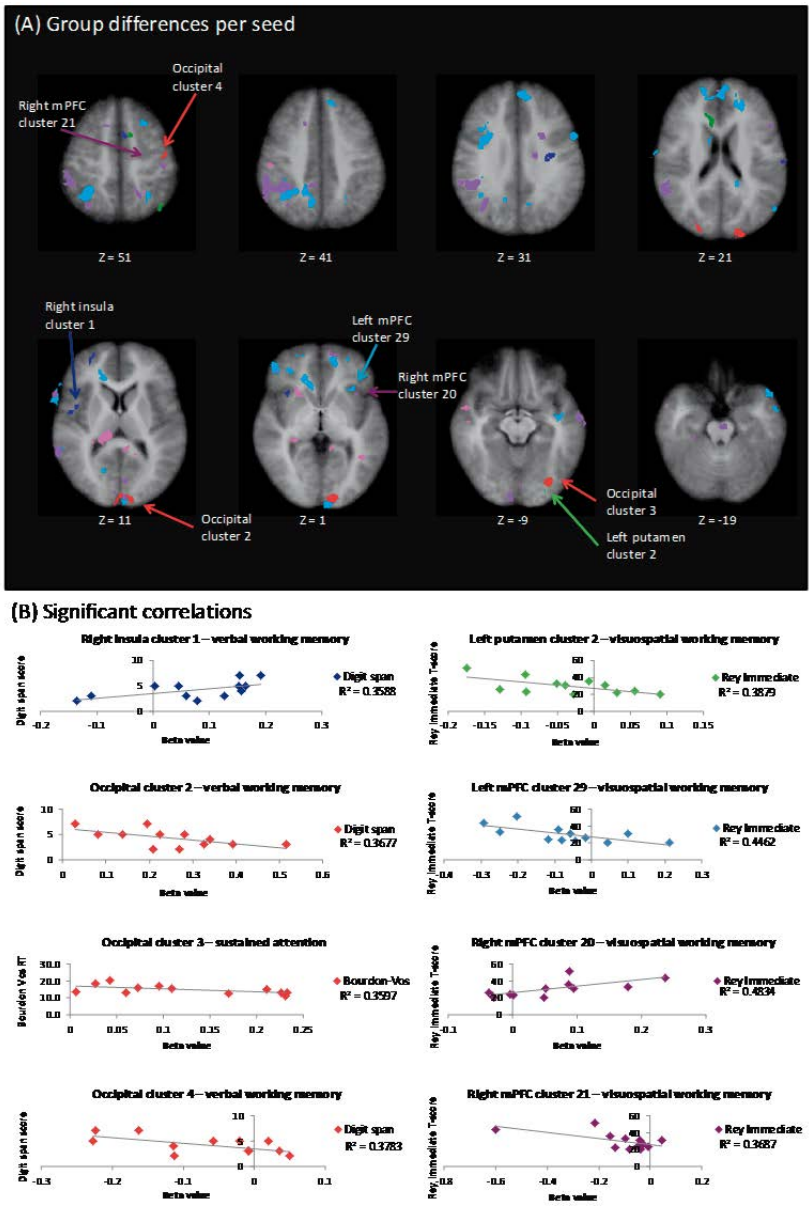

Figure 5: Clusters (VOI) of group differences.

Panel a: Overview of clusters (VOI) of group differences, specified per seed (see Table 3). Clusters with significant correlations to neurocognitive tests are marked. Color legend: purple = right mPFC seed, blue = left mPFC seed, pink = right putamen seed, light green = left putamen seed, green = left IFG seed, dark blue = right insula seed, dark red = left pre-SMA seed, red = occipital seed. Panel b: Scatterplots of clusters with significant correlations to visuospatial working memory (Rey Immediate Recall T-score; Meyers and Meyers 1995), verbal working memory (Digit Span score; Van Haasen et al. 1986) or sustained attention (Bourdon-Vos mean reaction time; Vos 1988). Note: though the right mPFC-left precentral gyrus with visuospatial working memory correlation might seem driven by two outliers, the correlation remains intact when outliers are removed.

The results from the right insula seed analysis support the claim of potentially disrupted sensory-motor integration in classic galactosemia patients too. We observed increased connectivity with the anterior insula and decreased connectivity with the posterior insula, as well as altered connectivity with other structures of the posterior insula network (PrG, PoG, pre-SMA) in patients. Our interpretation is based on previous studies that describe the insula – among other functions – as relevant for sensory-motor integration. More specific, the anterior insula has been associated with motor sequencing and speech planning (46), and the posterior insula has been connected to skeleto-body orientation (58).

The left IFG, which is known for its importance in language, demonstrated increased connectivity with left pre-SMA, and decreased connectivity with right ACC and left SPL. These findings might reflect aberrant communication between regions involved in language planning (IFG) and sensory-motor areas important for speech- and language production (pre-SMA, ACC, SPL). Note also that these results are in line with our previous task-based data, where the left IFG was also found to show decreased connectivity with superior parietal regions (in addition to other alterations) (26). The right IFG was not selected as seed for the analyses in the current study, however its connectivity was frequently altered in relation to selected seeds. This might point towards altered response inhibition or attentional control – representing important functions of the right IFG (59).

Evaluation of the occipital seed analysis showed decreased connectivity with bilateral cuneus, left lingual gyrus and left PrG in patients, and with the bilateral middle occipital gyrus as well (*q*(FDR) corrected maps). This further supports the description of altered visuospatial capacities in those patients, since these brain regions have been implicated in visuospatial attention/processing (cuneus, middle occipital gyrus (54, 60)) and motor output (PrG (61)). Findings from the right and left putamen analyses (altered connectivity with PrG, cerebellum) and the pre-SMA analysis (increased connectivity with SPL, which is involved in spatial orientation, locating objects and body parts in space, and visuospatial attention (50, 62)) are also in line with this hypothesis.

In addition to the importance for working memory and identification of landmarks and faces (63, 64), the lingual gyrus has been associated with lexical/semantic processing (65, 66). Abnormal connectivity of a seed to the lingual gyrus in patients was demonstrated across multiple analyses (right mPFC, left putamen and occipital seeds), which might point towards an important role for this structure in the pathogenesis of classic galactosemia in general, and specifically in the pathogenesis of language production problems observed in this disorder. Additionally, the STG and MTG, which are important for perception and production of language and speech (67), as well as for social cognition (68, 69), showed abnormal connectivity in patients across several analyses. Previous studies from our group already demonstrated specific abnormalities in the language network leading to lexical and syntactic planning impairments, in addition to often observed motor speech planning problems (12, 26).

To further investigate the relation between brain networks and behavior, we correlated the patients' resting-state brain connectivity patterns with their performances on several neurocognitive tasks. For that we used the individual neurocognitive test scores and the individual beta values per region (cluster). These beta values represent the quantification of functional connectivity of that region with the seed region. We selected only beta values from those clusters that were different between patients and controls. Although correlations could only be explored for the patient group, we observed some strong and specific correlations for several clusters. These correlations should be interpreted with caution, but they might support the general functional interpretation of our resting-state analysis. The observed correlations seem to underline a brain network-behavior relation, although this needs to be confirmed in task-based fMRI studies targeting these specific neurocognitive functions. Most importantly, brain-behavior correlations were detected especially for those clusters that come back across multiple seed analyses as being divergently connected (compared to controls), namely the lingual gyrus, PrG, insula and cuneus (Figure 5), thereby supporting the suggested relevance of certain connectivity problems in these patients.

In general, these findings might shed new light on the brain impairments in classic galactosemia. Future studies are needed to investigate whether the alterations observed in this study are the result of targeted damage to these specific regions/networks, or whether these structures are equally affected as other regions of the brain but show a more prominent functional impairment. Alternatively, these differences in functional connectivity might reflect compensation mechanisms to cope with cognitive difficulties. In order to provide more detailed hypotheses on this, investigations of longitudinal data and a systematic comparison of results of structural and functional brain imaging studies are needed.

The current findings are in line with earlier studies in the field of classic galactosemia. A previous tasked-evoked fMRI study from our group already demonstrated altered functional connectivity in the language network, with specific alterations at the level of the IFG, insula and pre-SMA, suggesting impaired sensory-motor integration, motor (speech) planning deficits and suboptimal communication between frontal regions and temporal/parietal regions (26). The connectivity patterns and network alterations are in agreement with the current resting-state findings. The observed language network abnormalities are also in line with an earlier syntactic language planning study using event related potentials (ERPs), in which lexical and syntactic planning impairments were seen (12). Results from an FDG-PET study pointed towards abnormalities of the superior temporal lobes, parietal regions, primary visual cortex, sensorimotor areas and frontal lobes (20), brain regions that had a repeatedly altered functional connectivity in the current study. Imaging studies exploring brain structure in patients with classic galactosemia provided evidence for both white and grey matter abnormalities in line with the neurocognitive profile of patients (27, 28) and with the here reported results.

One of the limitations of this study is its limited sample size, making this study of explorative nature. The small cohort size hampers solid conclusions on functional connectivity and confirmation of results in a larger sample is thus warranted. Moreover, genders were not equally balanced in our test sample (females>males) and this cohort is therefore not fully representative for the classic galactosemia population. Though cognitive performance does not seem to differ between male and female patients (16), future studies exploring potential gender variations in functional connectivity are desired. Furthermore, the current study method is based on certain assumptions and focuses on *a priori* selected seeds, deriving from previous investigations. Future resting-state fMRI studies could consider an independent component analysis approach, which analyzes the overall pattern of functional connectivity without focusing on specific regions of interest (70-72). The current SCA approach with 8 seeds also brings along multiple testing. Due to the nature of the current study, aiming to provide a first insight in functional brain networks at rest in this rare disorder, multiple comparisons corrections were not performed for group differences, which could lead to overestimation of effects. In addition, we correlated functional connectivity to results on neurocognitive tests in order to evaluate potential brain-behavior relations. Since correlations could only be explored for the patient group and multiple comparisons were not corrected, conclusions should be drawn with caution. These findings provide the first conjectures on potential brain-behavior relationships and the hypotheses generated by the current study require further testing in more dedicated study designs.

Taken together, robust differences in functional resting-state networks between patients with classic galactosemia and age- and gender-matched controls were found. Results point towards abnormalities in networks encompassing the mPFC, PrG, PoG and IPL, which are involved in sensory-motor integration and spatial orientation. Furthermore, altered connectivity of networks including the insula, IFG, pre-SMA, PrG, PoG and SPL, which are important for language production, sensory-motor integration and motor (speech) planning/sequencing, was demonstrated. Lastly, abnormalities were found in resting-state networks involving the occipital region, (pre)cuneus and lingual gyrus, which might be linked to the observed impairments in visuospatial capacities and working memory, and possibly lexical/semantic processing as well. Most importantly, across several seeds, altered functional connectivity to the lingual gyrus, the PrG, the insula and the cuneus was observed in patients, suggesting these brain regions might be of special importance and deserve more thorough investigations. In addition, these differences in network connectivity correlated with clinical test results in patients, supporting a relation between functional abnormalities and the clinical phenotype. Our findings contribute to the characterization of functional brain impairments in classic galactosemia, which is needed to create a better understanding of this enigmatic disease.

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Chapter 6

Bone health in classic galactosemia: systematic review and meta-analysis

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ABSTRACT

Introduction Previous studies have reported an association between classic galactosemia (CG) and decreased bone mass. The primary objective of this systematic review with meta-analysis was to determine the extent of bone mineral density (BMD) Z-score reduction. Low BMD was defined as a Z-score ≤-2 standard deviations (SD). The secondary objective was to evaluate other indicators of bone status through a descriptive analysis.

Methods Systematic search strategies were developed by an experienced clinical librarian. Selection of relevant manuscripts, risk of bias assessment and data-extraction were performed independently by two investigators.

Results Four studies were included in the meta-analysis. BMD Z-scores in children and adults with CG measured at the lumbar spine (LBMD; 4 studies; n=112), total hip (HBMD; 2 studies; n=58), and femoral neck (FBMD; 2 studies; n=73) were assessed. Mean BMD Z-scores in the CG population were: LBMD -0.70 (95% CI: -0.88, -0.52); HBMD -0.89 (95% CI: -1.14, -0.64); FBMD -0.63 (95% CI -1.29, 0.02). Results from studies included in the descriptive analysis (n=7) show that vitamin D levels were frequently in the low reference range, whereas serum calcium levels were within reference range.

Conclusion The mean BMD Z-score in the CG population is -0.7, which is lower than in the general population, though still within two SD of the reference mean of zero. This indicates that bone health is mildly affected in CG and that more patients, compared to the general population, are at risk for a BMD Z-score ≤-2 SD. In conclusion, clinicians should ensure appropriate preventive and therapeutic measures for CG patients.

INTRODUCTION

Classic galactosemia (CG, MIM 230400), a genetic disorder of galactose metabolism due to deficiency of galactose-1-phosphate uridyltransferase (GALT; EC 2.7.7.12), is characterized by the occurrence of late complications in spite of early diagnosis and lifelong dietary treatment. The first report of an association between CG and decreased bone mass dates back to 1993 (1) and a bone mineral density (BMD) Z-score more than two standard deviations (SD) below the mean was found in 25-30% of adult patients (2,3). Thorough evaluation of the frequency and severity of impaired bone health in CG patients, compared to the non-galactosemia population, is crucial for determining its extent and relevance for patient care.

CG patients could be at risk for compromised bone health due to diet restrictions, ovarian insufficiency in women, limited physical activity in some cases, and possibly unknown intrinsic factors associated with the disease. Sufficient intake of calories, protein and micronutrients is essential for acquiring an optimal bone mass, and the lifelong galactose restriction may predispose patients to nutritional deficiencies (3–6). Furthermore, ovarian damage resulting in low estrogen concentrations is present in over 80% of female patients with CG (7), which increases their susceptibility to the development of low bone mass. Remarkably, supplementation of calcium, vitamins and estrogen only seem to partially improve bone mass (1,8,9), which may point to the presence of an underlying intrinsic bone defect. Aberrant glycosylation of collagen and other glycoproteins related to bone metabolism, which is also seen in patients with other glycosylation defects such as phosphomannomutase 2 deficiency (PMM2-CDG) (10), has been suggested as a potential intrinsic abnormality (1,11) and the decreased IGF-I and IGFBP-3 concentrations that are found in patients might reflect this (12). Furthermore, reduced physical activity due to motor abnormalities (13) and cognitive impairment (14–17) may affect bone health as well. Clinical practice, focused on reducing the risk of impaired bone health in those with CG, includes routine monitoring of BMD, which is an important determinant of bone strength and has predictive value in assessing fracture risk (18), and optimization of exogenous factors affecting bone mass (nutrition, estrogen concentrations, physical activity)(19).

The primary aim of our study is to evaluate the extent of impaired bone health in patients with CG, and the need of monitoring and treatment. Few studies assessing bone health in CG are published. These studies have small patient sample sizes, and results vary from one study to another. As the current conjecture of low BMD in CG is based on single studies, further evaluation is needed to confirm the validity of this hypothesis (20). Meta-analysis is the most commonly used statistical technique to pool results from two or more separate studies. The added value of a meta-analysis may include increased power and improved precision of the results. Therefore, we performed a systematic review with meta-analysis of BMD in children and adults with CG. As recommended by the International Society for Clinical Densitometry (ISCD), low BMD

is defined here as a BMD Z-score ≤ -2.0 SD (21,22). We also explored the usefulness of other indicators of bone status as potential diagnostic or monitoring tools.

METHODS

Research question

The primary outcome for our meta-analysis was bone mass reported as BMD (areal BMD), either as Z-score or as absolute measurement, assessed with dual energy X-ray absorptiometry (DXA) since this is the preferred tool for evaluating BMD/bone mass in both children and adults (21,22). BMD is a major determinant of bone strength and its assessment is considered the cornerstone in the diagnostics of low bone mass (23). Only areal BMD measurements were included in the analysis since these are most commonly used in clinical practice; results of estimated volumetric BMD were not included.

The secondary outcomes for our systematic review, reported in a descriptive way, were bone mineral content (BMC), parameters involved in bone metabolism such as vitamins, minerals, hormones, bone turnover markers (BTM), and fracture risk.

Inclusion and exclusion criteria for study selection

We included all original studies (cross-sectional study design, randomized controlled trial [RCT], cohort study, case-control study) as well as conference abstracts on bone health in children and adults with CG. If there was (potential) overlap between study cohorts, only the first/original article was included. Articles in a language other than English, and animal and cell studies were excluded.

Search strategy

A computerized literature search was conducted in MEDLINE, EMBASE and the Cochrane Library by one of the investigators (LW) and a trained clinical librarian from University of Amsterdam (see Supplementary data S1, Search strategies). Databases were searched initially in February 2015 and a final search was completed in July 2015 to ensure the inclusion of recently published articles. No limits were used in these searches.

Study selection

Titles and abstracts generated by literature searches were screened by two separate researchers (BvE and LW) to select potentially eligible studies. Studies not relating to

the research question were excluded. Selected conference abstracts and full text articles of selected studies were independently reviewed by two authors (BvE and LW) for inclusion in the systematic review. In case of exclusion, the reason was reported. Additionally, reference lists from included trials and excluded narrative or systematic reviews were hand-searched to identify additional relevant studies. Consensus was reached between the two authors regarding the eligibility assessments.

Data extraction

Data were extracted by two separate researchers (BvE and LW) according to predefined criteria (see Supplementary data S2a and S2b, Summary of evidence tables). Outcome measures of interest were BMD at any site measured with DXA; BMC; incidence of fractures; BTM reflecting bone formation (bone-specific alkaline phosphatase, undercarboxylated osteocalcin [ucOC], carboxylated osteocalcin [cOC]), bone resorption (N-terminal telopeptide, C-terminal telopeptide) or bone modeling (insulin-like growth factor-1 [IGF-1]); and vitamins (vitamin D), minerals (calcium) and hormones (estradiol, parathormone) important for bone metabolism.

Data collection processes

If a mean BMD Z-score and/or standard deviation (SD) was not reported for the entire cohort in an article, the authors were requested to share either these variables together with mean age at testing and SD, or individual patient data (IPD). IPD included individual mean BMD Z-score with SD, age at testing and gender. The corresponding author was contacted first, and if there was no reply within four weeks, one of the other authors was contacted.

Individual patient data integrity

IPD were checked for integrity by reviewing completeness; in case of incompleteness the data were not included in the systematic review. If more data were received from the authors than originally published, these were only included if patient characteristics matched those from the original article. In case of any discrepancies, the authors were contacted.

Assessment of quality and risk of bias in individual studies

Quality appraisal and assessment of bias were performed with an appropriate checklist from the 'Scottish Intercollegiate Guidelines Network' (SIGN), if available (available for: RCT, non-RCT, cohort study, case-control study). Quality appraisal and risk of bias analysis were performed and discussed by two independent investigators (BvE and LW).

Articles were appraised as low, acceptable or high quality; those assessed as low quality were excluded from the review.

Data analysis

We performed a meta-analysis of BMD to evaluate whether the mean BMD Z-score in the CG population differs from normative data (a mean BMD Z-score of zero in the general population). We used Review Manager 5.3 version 5.3.5 for the analysis. The inverse-variance method was used to pool study data, and the individual effect sizes were weighted according to the reciprocal of their variance (24). I^2 was used as a measure of heterogeneity (25). The p-value used to reject the null hypothesis of homogeneity was 0.1 (p-value of Q; Q=chi squared statistic). In case of low heterogeneity (0-30%) a fixed effects model was used to pool data. In case of moderate to high heterogeneity (30-100%), both a fixed and random effects model were applied, resulting in a sensitivity analysis with description of differences between the fixed and random effects models and selection of the most appropriate model. Aggregate data and IPD were analyzed together using a two-stage approach: for IPD, a mean BMD Z-score and SD were calculated first, and were then included in the next step as aggregate data. BMD Z-scores were pooled based on the measurement site (lumbar spine, total hip, femoral neck and total body). For the overall effect size, a p-value of 0.05 was considered statistically significant. Yet, in case of multiple testing, adjustment for Bonferroni correction was applied.

In order to assess clinical relevance of the mean BMD Z-score of the CG population, the normal distribution was used to find the percentiles of Z-scores, and thus the estimated proportion of patients with a BMD Z-score ≤-2 SD (low bone mass) using SPSS version 23. For this, two approaches were used, one with the SD of normative data (mean of zero, SD 1), another with the SD of the mean calculated in our meta-analysis.

The secondary outcome measures (BMC, minerals, vitamins, hormones and BTM) were presented in a descriptive manner.

RESULTS

Study selection

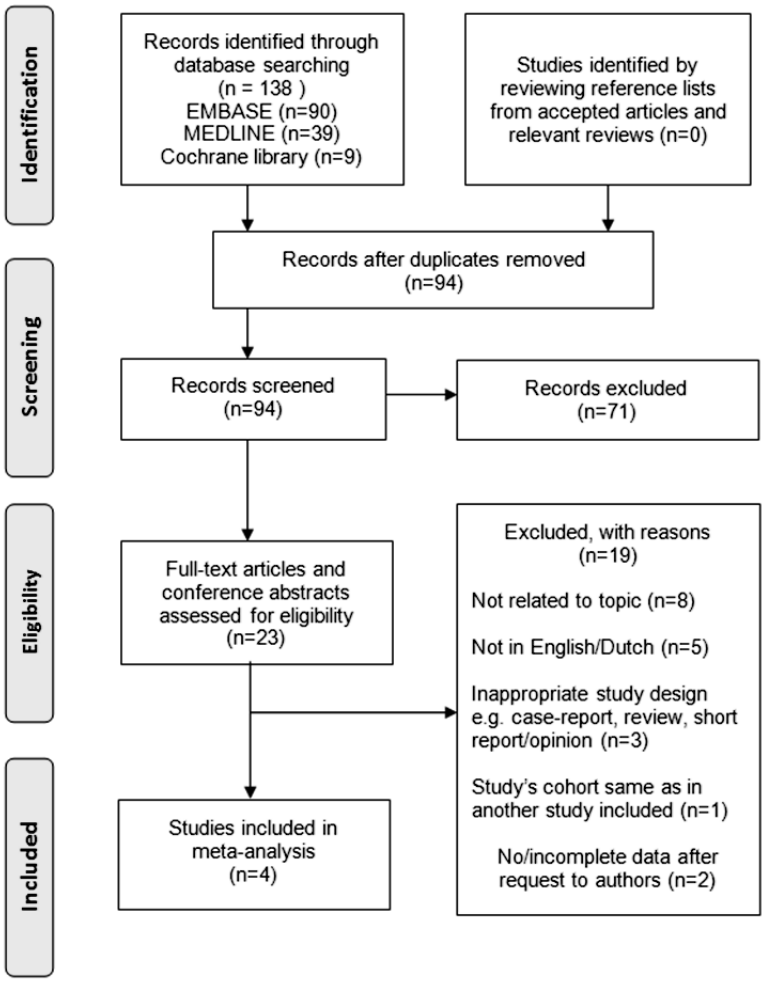
The literature search identified 138 potential publications of which 94 remained after removing duplicates (n=44). After screening the titles and abstracts of the unique publications, 71 were excluded because they did not relate to the research question. Detailed evaluation of the 23 selected publications led to the exclusion of 10 studies for various reasons (see Figure 1: Flow diagram selection process).

For the meta-analysis, eight studies were selected because they encompassed BMD measurements. Only one of these studies reported all data required for inclusion (26);

the authors of the other 7 studies were contacted for additional information. Three authors provided the required data at request (3,27,28)(Supplementary data S3, Data collection process). Accordingly, a total of four articles were included in our meta-analysis on BMD.

A total of seven studies reported on our secondary outcome measures and were therefore included in the descriptive analysis.

The complete study selection process is presented as a flow diagram, separately for the meta-analysis and the descriptive analysis (Figures 1a and 1b).



From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

Figure 1a: Flow diagram selection process meta-analysis.

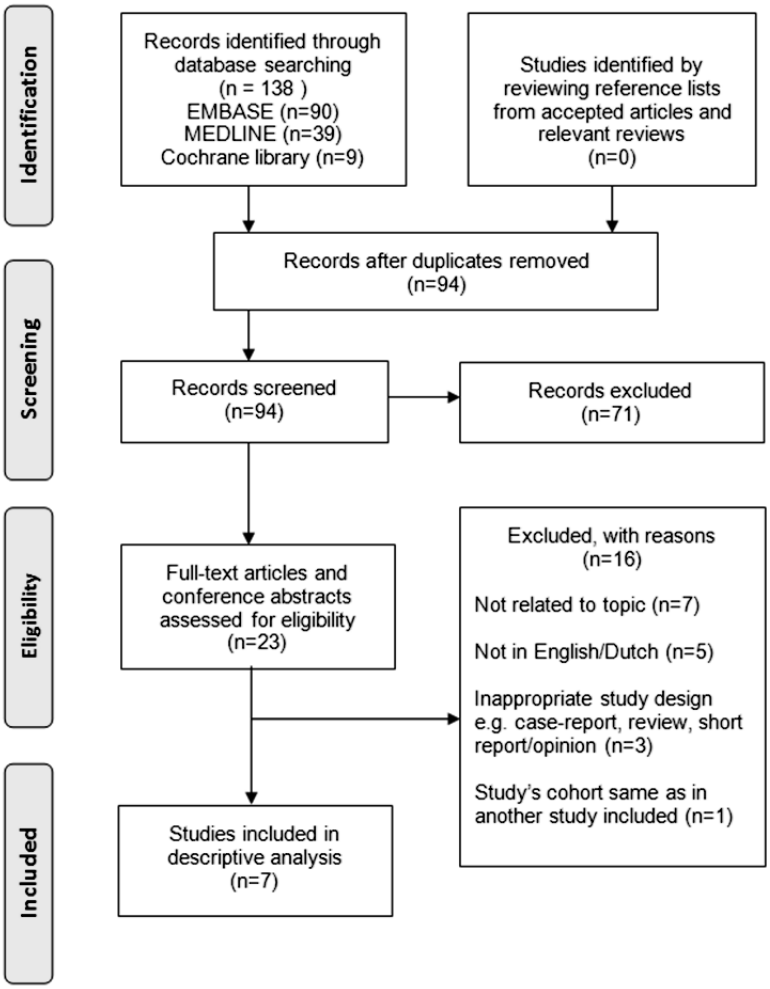


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Figure 1b: Flow diagram selection process descriptive analysis.

Included studies

Characteristics of the studies included in the meta-analysis or descriptive analysis are presented in Supplementary data S2a and S2b, Summary of evidence tables. Age of the patients in the included studies ranged from 2.5-59 years.

Individual patient data integrity

No issues with IPD integrity were detected.

Assessment of quality and risk of bias in individual studies

A quality appraisal with the standardized checklist was applicable for only one study, a randomized controlled trial (8). The paper was graded high quality as defined by the risk of bias checklist for randomized-controlled trial of SIGN.

Meta-analysis on BMD in classic galactosemia patients

Four studies (three cross sectional studies and one retrospective case series) were included in this meta-analysis of BMD Z-score (3,26–28). Panis et al. (2004) assessed 40 patients with a mean age of 8.9 years (range 3.0-17.3). Waisbren et al. (2012) evaluated BMD in 33 patients with a mean age of 32.6 years (range 18-59). Doulgeraki et al. (2014) reported BMD Z-scores of 14 patients with a mean age of 13.16 years (range 6.17-16.58 years). Tan et al. (2014) provided the individual patient data reported in their conference abstract as well as additional new data (n=5). The mean age in these 25 patients was 13.5 years (range 5-41 years). Mean BMD Z-scores were calculated and are presented in Supplementary Data S2a.

Complete cohort (children and adults)

A total of 112 CG patients assessed in 4 studies were included in this part of the meta-analysis. At the site of the lumbar spine, a mean BMD Z-score of -0.70 (95% CI: -0.88, -0.52) was found (3,26–28). Heterogeneity was zero (I^2 = 0%); a fixed effects model was used. The overall effect was statistically significant (p < 0.00001) when compared to the mean of normative data (BMD Z-score of zero). One of the studies crossed the line of no effect (Figure 2).

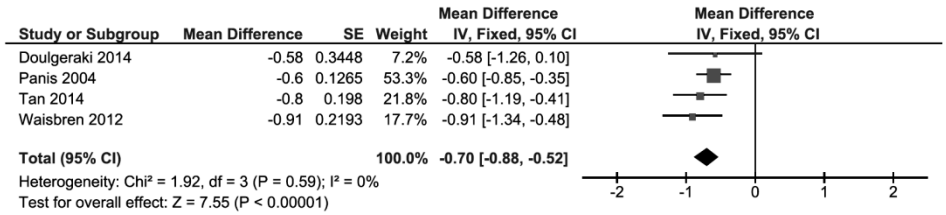

Figure 2: Forest plot of lumbar spine BMD Z-score in complete CG cohort.

In 58 pooled patients included in 2 studies, mean total hip BMD Z-score was -0.89 (95% CI: -1.14, -0.64)(3,27). Heterogeneity was zero (I^2 = 0%); a fixed effects model was used. The overall effect was significantly different from the mean of normative data (p < 0.00001) and none of the studies crossed the line of no effect (Figure 3).

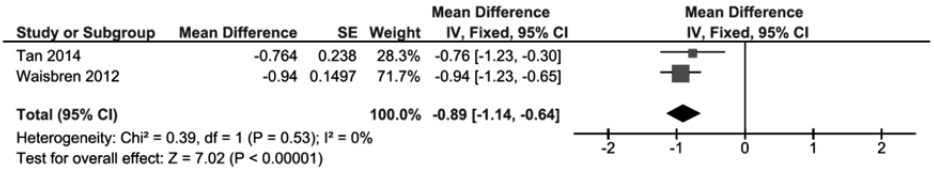

Figure 3: Forest plot of total hip BMD Z-score of the complete CG cohort.

At the site of the femoral neck, 73 CG patients from 2 studies were pooled (3,26). The heterogeneity was high (I^2 = 91%), leading to determination of the mean BMD Z-score with both a fixed as well as a random effects model (Figures 4 and 5, respectively). A mean BMD Z-score of -0.63 was found with both models, with a difference in 95% confidence interval (95% CI fixed effects model: -0.83, 0.44; 95% CI random effects model: -1.29, 0.02). As heterogeneity was very high and both studies are of adequate quality with comparable cohort sizes, it was chosen to base conclusions on the results of the random effects model. Even though both studies did not cross the line of no effect, the overall effect was not significantly different from the mean of normative data when using the random effects model (p = 0.06) (Figure 5).

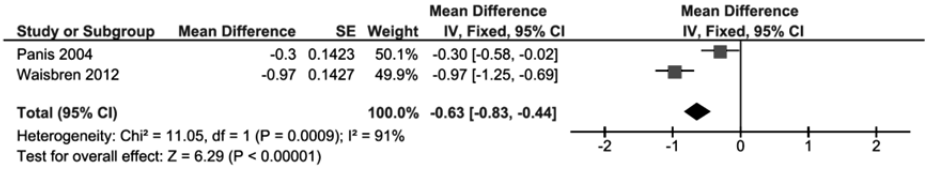

Figure 4: Forest plot of femoral neck BMD Z-score in complete CG cohort (fixed effects model).

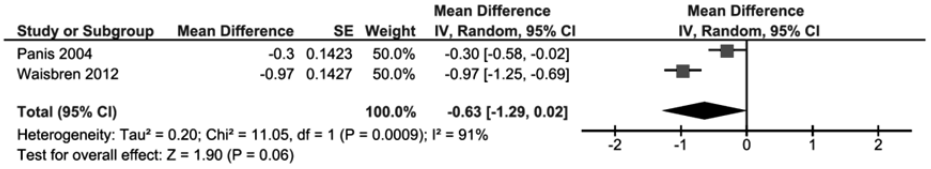

Figure 5: Forest plot of femoral neck BMD Z-score in complete CG cohort (random effects model).

Children with CG

In pooled data from 76 children from 3 studies, mean lumbar spine BMD Z-score was -0.64 (95% CI: -0.84, -0.43)(26–28). Heterogeneity was zero (I^2 = 0%); a fixed effects model was used. This was overall significantly different from the mean of normative data (p < 0.00001). One study crossed the line of no effect (same study as in lumbar spine BMD Z-score analysis of the entire patient group) (Figure 6).

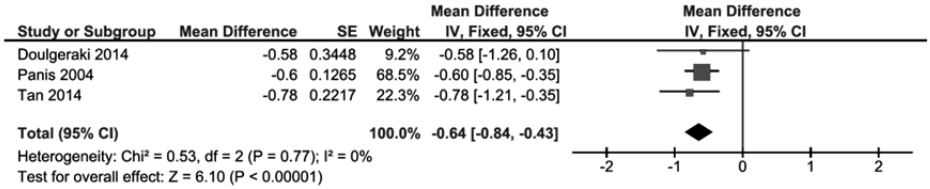

Figure 6: Forest plot of lumbar spine BMD Z-score in children with CG.

Adults with CG

In pooled data from 36 adults with CG included in 2 studies, mean lumbar spine BMD Z-score was -0.94 (95% CI: -1.30, -0.57)(3,27). Heterogeneity was zero (I^2 = 0%); a fixed effects model was used. The overall effect was statistically significant from the mean of normative data (p < 0.00001), and none of the studies crossed the line of no effect (Figure 7).

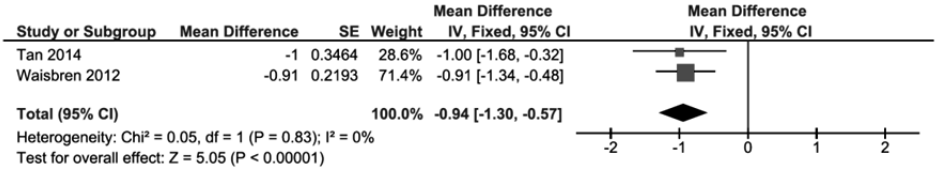

Figure 7: Forest plot of lumbar spine BMD Z-score in adults with CG.

Mean BMD Z-score at the total hip in this group of adults was -0.91 (95% CI: -1.20, -0.63)(3,27). Heterogeneity was zero (I^2 = 0%); a fixed effects model was used. One study crossed the no effect line, but the overall effect was significantly different from the mean of normative data (p < 0.00001) (Figure 8).

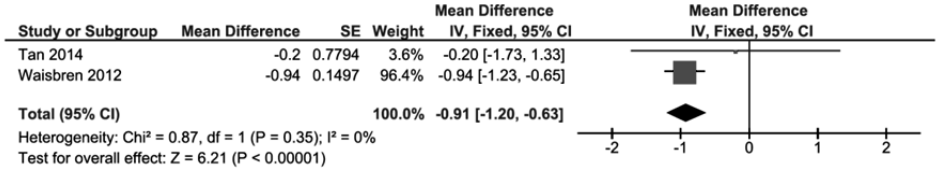

Figure 8: Forest plot of total hip BMD Z-score in adults with CG.

We could not perform a subgroup analysis on BMD Z-scores for adult males and adult females separately since one study included data on only one or two patients.

Clinical relevance

The normal distribution was used to find the percentiles of Z-scores, and thus the estimated proportion of patients with a BMD Z-score ≤-2 SD (low bone mass). Two approaches were used: one with an SD of 1.9 (resulting from the 95% CI of the calculated mean lumbar spine BMD Z-score of -0.7 in the complete CG group) and one with an SD of 1.0 (according to the normal distribution curve of BMD in the general population). Accordingly, 10-25% of CG patients are estimated to be at risk for a BMD Z-score ≤ -2 SD, and thus a low bone mass, whereas this is only 2.3% in the general population (normally distributed parameter).

Descriptive analysis

Other indicators of bone status in classic galactosemia

Bone mineral content

Another indicator of bone health is BMC, which reflects other aspects of bone mass acquirement than BMD and might therefore be of additional value in children (29). BMC of femoral neck, lumbar spine and total body was assessed in a single study by Panis et al. (2006) (40 patients, age range 3-17 years)(8). In this randomized-controlled trial, in which the effect of supplementation of calcium, vitamin K1 and vitamin D3 on bone health was studied, baseline measurements of BMC Z-scores in 40 children with CG (age 3-17 years) were conducted. Mean BMC Z-scores (varying between -0.3 and -1.1 for different subgroups) were lower than in controls.

Vitamins, minerals and hormones

Six studies evaluated vitamin D status in CG patients (n=197)(3,26,30–33). One study assessed 1,25-OH-D concentrations only (26), four measured 25-OH-D only, and one evaluated both (32). The studies varied with regard to vitamin D reference ranges and units, and in some the specified range of desired vitamin D concentrations was not stated. Two studies in adults with CG reported that most patients had levels in the low reference range (3,33). Compliance to vitamin D supplements differed between the two cohorts. The remaining four studies, performed in children with CG, found vitamin D levels to be within reference range.

Four studies with a total of 148 patients assessed serum calcium levels (26,30–32). In all studies calcium levels were found to be within reference range. Rubio-Gozalbo et al. (2002) found no correlation between BMD results and calcium intake in their cohort of children with CG (32).

Parathormone was also measured in the studies by Rubio-Gozalbo et al. (2002) and Panis et al. (2004) and revealed values within reference range. Only one study meas-

ured 17-beta estradiol levels in a cohort of 40 children aged 3-17.3 years (mean age 8.9 years)(26). Mean levels did not differ from reference values.

Bone turnover markers

Four cross-sectional studies examined BTM in CG patients (26,30–32), three studies included children only (<18 years) and one study included patients up to 20 years. A total of 148 patients were included in these studies (range 11-62 patients/study). There were no studies reporting on markers of bone status in adult patients.

Carboxy-terminal telopeptide of type 1 collagen, measured in all four studies, was found to be significantly reduced in children but not in adolescents when compared to controls. Panis et al. (2004) found it to be inversely correlated with BMD Z-score of the femoral neck and lumbar spine (26). Amino terminal telopeptide of type I collagen levels were measured in two study cohorts and levels were found to be significantly low in both cohorts of CG children (26,32). Gajewska et al. (2008) reported about 30% higher values of bone-specific alkaline phosphatase in adolescents than in controls (31), whereas normal values were found in children. In addition, Gajewska et al. found increased osteocalcin (OC; sum of carboxylated [cOC] and under-carboxylated osteocalcin [ucOC]) levels in adolescents with CG (31), whereas Panis et al. (2004) reported significantly decreased cOC levels with normal ucOC levels in children (26). Normal values of OC (30,31) and cOC and ucOC (32) in children were reported in three studies. Furthermore, Panis et al. (2004), the only study measuring IGF-1 levels, reported reduced IGF-1 Z-scores in children (26) and found that IGF-1 Z-score was a strong positive predictor of femoral neck and lumbar spine BMD.

Fracture history

Waisbren et al. (2012) reported that 45% of 33 patients (mean age 32.6 years, range 18-59) had broken a bone, six during childhood and the others at ages 20 to 46 years (3).

DISCUSSION

In this systematic review we evaluated bone mass in patients with CG through a meta-analysis of BMD. The results of our meta-analysis indicate that mean bone mass in the CG population, reflected by mean BMD Z-score, is more than a half-standard deviation lower than in the general population. While this is still within two SD of the normative mean, this result indicates that an increased proportion of individuals with CG will have a BMD Z-score ≤ -2 SD, and thus a low bone mass for age, as compared to individuals in the general population. Based on the meta-analysis, estimated prevalence of BMD Z-score ≤-2 SD in patients with CG is 10% to 25%, which is higher than in the general population (2.3%). Mean BMD Z-scores in pooled data of adults and children with CG were reduced at all sites (lumbar spine -0.70, total hip -0.89, femoral neck -0.63), of which

only the latter was not statistically significant, probably due to heterogeneity between included study cohorts. Findings from our descriptive analysis support the need for improved evaluation and optimization of vitamin D concentrations. Though serum calcium levels were within reference range in all studies that addressed calcium status, ensuring sufficient intake of calcium remains a point of attention in this population. Data on the role of BMC and BTM as other indicators of bone status are very limited and, in the case of BTM, highly ambiguous. Therefore, routine screening of these indicators in CG patients does not seem of additional value at present. Literature on bone mass in CG is scarce and study cohorts are small as a result of the rarity of the disease. This limits the number of studies and patients included in our meta-analysis, which hampers extensive subgroup analyses and solid conclusions. However, this systematic review is currently the most comprehensive study evaluating bone health in CG patients, thereby providing results that are more representative for the whole population of CG patients.

This study was limited in that not all original patient data could be obtained. In addition, data used in this study were all cross-sectional, and conclusions about progression over time must therefore be interpreted with caution. Longitudinal studies following patients in time are needed to enable firm conclusions on this. Moreover, ISCD recommendations for pediatric DXA (34) were not unanimously followed by the researchers, as they used different sites of measurement and did not take into account the presence of short stature or growth delay, with the exception of only one study (28). Furthermore, data on fracture history were obtained in only one study (3), though this is required to establish a diagnosis of osteoporosis. Future studies should consider the ISCD recommendations to further improve insights on bone health in CG.

CONCLUSIONS

BMD Z-scores in individual CG patients are within two SD of the normative mean in the majority of patients with CG. However, results from our meta-analysis demonstrate that the mean BMD Z-score in the CG population is lower than the mean BMD Z-score in the general population. These results suggest that bone health in general is mildly affected, and that an estimated proportion of 10-25% of patients with CG could be at risk for a low bone mass (BMD Z-score ≤-2) as compared to the general population. With the currently available literature, which lacks data on fracture prevalence, it is impossible to draw conclusions about osteoporosis risk. Vitamin D levels are low in many patients, emphasizing the need for monitoring of 25(OH)D levels and vitamin D supplementation. Optimization of calcium intake remains important. Evaluation of the importance of other parameters of bone health (BCM, BTM, hormones) was inconclusive due to a limited number of studies with inconsistent results. Concluding, it is important that treating physicians are aware that patients with CG are at risk for having or developing low bone mass, so that patients will be screened and treated appropriately.

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SUPPLEMENTARY DATA S1: SEARCH STRATEGIES

Searches in all databases were performed in February 2015.
Search strategy MEDLINE

1	galactosemias/
2	(galactosem* or ((galt utp hexose 1 phosphate uridyltransferase or gale or galk or galactokinase) adj3 deficien*)).tw.
3	1 or 2
4	Bone Density/
5	Bone Diseases, Metabolic/
6	exp bone resorption/
7	exp "Bone and Bones"/
8	(Calcium/ or vitamin d/ or vitamin k/) and (me or bl).fs.
9	Absorptiometry, Photon/
10	Osteocalcin/
11	((bone adj3 density) or bone mass or fractures or fracture or bone metabolism or bone resorption or osteolysis or bone loss or bone mineral* or osteoporosis or osteolysis or skeletal health or bone turn over or dxa or dual energy x ray).mp,kf.
12	exp Fractures, Bone/
13	or/4-12
14	3 and 13

Search strategy EMBASE

1	galactosemias/
2	(galactosem* or ((galt utp hexose 1 phosphate uridyltransferase or gale or galk or galactokinase) adj3 deficien*)).tw.
3	1 or 2
4	Bone Density/
5	Bone Diseases, Metabolic/
6	exp bone metabolism/
7	exp "Bone and Bones"/
8	Calcium/ec or vitamin d/ec or vitamin k/ec
9	Absorptiometry, Photon/
10	Osteocalcin/
11	((bone adj3 density) or fracture or fractures or bone mass or bone resorption or bone metabolism or bone loss or bone mineral* or osteoporosis or osteolysis or skeletal health or bone turn over or dxa or dual energy x ray).mp,kw.
12	exp Fractures, Bone/
13	calcium blood level/
14	or/4-13
15	3 and 14

Cochrane Library

Search in Title, Abstract, Keywords: galactosemias (MeSH term)

SUPPLEMENTARY DATA S2A: SUMMARY OF EVIDENCE TABLE PRIMARY OUTCOME (BMD)

Study (first author, year)	Study design	Study population			Test used	Outcomes	Compared to	Results
		Number	Sex	Age				
Doulgeraki 2014	Cross-sectional study	14	8 male 6 female	13.16 (6.17-16.58) yrs	DXA	BMD Z-score Lumbar spine, total body	General population	BMD Z-scores (median, range): Lumbar spine: -0.65 (-2.5 to 1.4) Total body: -0.3 (-1.7 to 0.6) Overall, median BMD Z-scores within normal range. 5 patients low-normal lumbar spine Z-score (between -1 and -2), whereas total body Z-scores were normal. ***BMD Z-scores (mean±SD)*** *Lumbar spine: -0.58 ±1.29* *Total body: -0.6 ±0.9*
Panis 2004	Cross-sectional study	40	13 male 27 female	3.0-17.3 yrs Mean: 8.9±4.1 yrs	DXA	Absolute BMD, BMD Z-score Lumbar spine, femoral neck	General population	**BMD Z-score femoral neck (mean±SD):** -0.3±0.9 (-1.6 to 1.4) **BMD Z-score lumbar spine (mean±SD):** -0.6±0.8 (-2.2 to 1.4) Mean BMD Z-score of lumbar spine significantly decreased when compared to reference data (p<0.001).
Rubio-Gozalbo 2002	Cross-sectional study	11	5 male 6 female	2.5-18.0 yrs	DXA	BMD Z-score Total body	General population	**BMD Z-score total body (mean, range):** -0.99 (-0.5 to -1.4) (p<0.05).

Study (first author, year)	Study design	Study population			Test used	Outcomes	Compared to	Results
		Number	Sex	Age				
Tan 2014	Retrospective case series (conference abstract)	25	15 male 10 female	5-41 yrs Mean: 13.5±7.1 yrs	DXA	BMD Z-score Lumbar spine, total hip	General population	70% of patients >10 yrs of age had a normal bone density. ***BMD Z-score entire cohort (mean±SD):*** *Lumbar spine: -0.80 ±0.99* *Total hip: -0.76 ±1.19* ***BMD Z-score children (mean±SD):*** *Lumbar spine: -0.78 ±1.04* *Total hip: -0.84 ±1.18* ***BMD Z-score adults (mean±SD):*** *Lumbar spine: -1.00 ±0.60* *Total hip: -0.20 ±1.35* ***BMD Z-score adult males (mean±SD):*** *Lumbar spine: -0.70 ±0.42* *Total hip: 0.35±1.35*

Study (first author, year)	Study design	Study population			Test used	Outcomes	Compared to	Results
		Number	Sex	Age				
Waisbren 2012	Cross-sectional study	33	17 male 16 female	18-59 yrs Mean: 32.6±11.7 yrs	DXA	BMD Z-score Hip, femoral neck, spine	General population	Bone density on average greater than one SD below the mean (Z-score= -1.1). 8/33 (24%) had Z-score greater than 2 SD below the mean. 15 subjects (45%) had history of fractures; 6 during childhood, 9 at ages 20-46 yrs. ***BMD Z-score adults (mean±SD):*** *Lumbar spine: -0.91 ±1.26* *Total hip: -0.94 ±0.86* *Femoral neck: -0.97 ±0.82* ***BMD Z-score males (mean±SD):*** *Lumbar spine: -0.80 ±1.28* *Total hip: -0.81 ±0.70* *Femoral neck: -0.89 ±0.66* ***BMD Z-score females (mean±SD):*** *Lumbar spine: -1.03 ±1.27* *Total hip: -1.08 ±1.01* *Femoral neck: -1.05 ±0.97*

SUPPLEMENTARY DATA S2B: SUMMARY OF EVIDENCE TABLE SECONDARY OUTCOMES (BMC, BTM, VITAMINS, MINERALS AND HORMONES)

Abbreviation list

1.25OHD = 1,25-dihydroxy vitamin D
25-OH-D vitamin = 25-hydroxycholecalciferol
AP = alkaline phosphatase
BAP = bone-specific alkaline phosphatase
Ca= calcium
cOC = carboxylated osteocalcin
CTX = carboxy-terminal telopeptide of type 1 collagen
ERT = estrogen replacement therapy
IGF-1 = insulin-like growth factor-1
Mg = magnesium
NA = not applicable
NTX = amino terminal telopeptide of type I collagen
OC = osteocalcin
P = phosphate
PTH = parathormone
ucOC =under-carboxylated osteocalcin
Z= zinc

Study (first author, year)	Study design	Study population			Outcomes	Compared to	Results
		Number	Sex	Age			
Gajewska 2006	Cross-sectional study	35	17 male 18 female	1-10 yrs Mean: 5.0 yrs	Serum Ca, P, 25(OH)D, AP, OC, BAP, CTX	Control group OC/BAP/CTX: 55 healthy children, 30 male/25 female, 1-10 yrs (mean 7.0). Other outcomes compared with normal reference range.	CTX 20% lower than controls, p<0.001. All other values similar to controls or within reference range.

Study (first author, year)	Study design	Study population			Outcomes	Compared to	Results
		Number	Sex	Age			
Gajewska 2008	Cross-sectional study	62	32 male 30 female	2-20 yrs Group children: N=32 17 male 15 female 2-9 yrs Group adolescents: N=30 15 male 15 female 10-20 yrs	Serum Ca, P, 25(OH)D, OC, BAP, CTX-I	70 healthy controls, 35 male/35 female Group children: N=40 20 male 20 female Age 6.4±2.6 yrs Group adolescents: N=30 15 male 15 female Age 15.6±2.4 yrs	Substantial overlap in patients with 2006 study. CG children: CTX-I is about 20% lower than in controls ($p<0.001$). BAP and OC not significantly different. CG adolescents: BAP is about 30% higher than in controls ($p<0.05$), OC is about 35% higher than in controls ($p<0.02$). CTX-I not significantly different. Healthy adolescents have significantly lower levels of CTX-I, BAP and OC than healthy children. Ca, P, 25(OH)D in CG patients within normal ranges.
Panis 2004	Cross-sectional study	40	13 male 27 female	3.0-17.3 yrs Mean: 8.9±4.1 yrs	Serum Ca, P, Mg, Zn, 1.25OHD, PTH, 17-beta estradiol, IGF-1 Z-score, CTX, BAP, cOC, ucOC, ucOC/cOC ratio, NTX	Reference values	Significantly decreased: cOC, NTX, CTX, IGF-1 Z-scores. All other outcomes within reference range.

Study (first author, year)	Study design	Study population			Outcomes	Compared to	Results
		Number	Sex	Age			
Panis 2006	Randomized-controlled trial on effect of calcium, vitamin K1 and D3 supplementation on bone mineral content	40	13 male 27 female	3-17 yrs	DXA	BMC Femoral neck, lumbar spine, total body	Pediatric reference data
Rubio-Gozalbo 2002	Cross-sectional study	11	5 male 6 female	2.5-18.0 yrs	Serum Ca, P, PTH, AP, 25(OH)D, 1.25OHD, cOC, ucOC, NTX, BAP	Ca/P/PTH/AP/vit D compared to reference range Other: 19 healthy controls, age and sexed matched	NTX significantly lower than in controls ($p<0.001$). There was no correlation between BMD results and dietary calcium intake.
Sirrs 2010	Retrospective case series (conference abstract)	16	9 male 7 female	17-55 yrs Mean: 26.6±9 yrs	25(OH)D	Reference range: <40 nmol/L deficient 40-79 nmol/L insufficient >80 nmol/L sufficient	9 patients had 25(OH)D levels in insufficient range, 1 patient in deficient range, despite half of them having moderate-good compliance to supplements (total daily vitamin D intake ≥400 IU).
Waisbren 2012	Cross-sectional study	33	17 male 16 female	18-59 yrs mean: 32.6±11.7 yrs	Plasma 25(OH)D	Reference range	80% has 25(OH)D level below the sufficient range. Mean plasma 25(OH)D 27±11 ng/mL, reference range 32-100 ng/mL.

SUPPLEMENTARY DATA S3: DATA COLLECTION PROCESS BMD Z-SCORES

Study (first author, year)	Study design	Information on BMD Z score	Authors contacted	Data received	Data included in meta-analysis
Burlina et al. 2014	Retrospective case series (conference abstract)	No	Yes	No	No
Coss et al. 2013	Retrospective case series	No	Yes	Yes, individual patient data	No, many missing data and possible selection bias
Doulgeraki et al. 2014	Cross-sectional study	Median BMD Z-score and range	Yes	Yes, mean Z scores and SD	Yes
Karadag et al. 2013	Retrospective case series	No	Yes	No	No
Panis et al. 2004	Cross-sectional study	BMD Z-score , SD and range	- (data complete for meta-analysis)	-	Yes
Rubio-Gozalbo et al. 2002	Cross-sectional study	BMD Z-score and range, no SD	Yes	No, data not available	No
Tan et al. 2014	Retrospective case series (conference abstract)	No	Yes	Yes, individual patient data	Yes
Waisbren et al. 2012	Cross-sectional study	No	Yes	Yes, individual patient data	Yes

Chapter 7

Revised proposal for the prevention of low bone mass in patients with classic galactosemia

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ABSTRACT

Decreased bone mass is frequently encountered in classic galactosemia, an inborn error of galactose metabolism. This decrease is most prominent in adults, but is already seen in prepubertal children with increased risk of osteoporosis and fractures later in life. Therefore, bone health in patients with classic galactosemia is increasingly monitored. Although the pathophysiological mechanism is still not fully understood, several factors could negatively affect bone metabolism in this disease. Patients are at risk of nutritional deficiencies due to the galactose-restricted diet. Primary ovarian insufficiency (POI) in female patients also contributes to decreased bone mass. Furthermore, patients with classic galactosemia might be less physically active due to motor or neurological impairments. A disease specific intrinsic abnormality has been suggested as well. This revised proposal is an update of the 2007 recommendations. In this current approach we advise comprehensive dietary evaluation, optimization of calcium intake if needed, monitoring and if necessary supplementation of vitamin D, hormonal status evaluation and hormone replacement therapy (HRT) consideration, as well as a regular exercise and assessment of skeletal deformities and clinically significant fractures. We propose bone mineral density (BMD) assessment by serial DXA scans of lumbar spine, femoral neck and total hip in adults and lumbar spine and total body less head (TBLH) in children.

INTRODUCTION

Decreased bone mass is frequently encountered in classic galactosemia (OMIM 230400), an inborn error of galactose metabolism (1-7). This decrease in bone mass is most prominent in adults, but is already seen in prepubertal children (4-6). Since bone mass increases quickly during puberty and reaches its peak at the end of adolescence or early adulthood, a low bone mass at an early age predisposes to osteoporosis and an increased risk of fractures later in life (8). Therefore, bone health in patients with classic galactosemia is increasingly monitored.

Scarce data are available with regard to reduced bone mass and its clinical relevance in this patient population. The average reported bone mineral density (BMD) as assessed by dual-energy X-ray absorptiometry (DXA) in adults with galactosemia is more than one standard deviation (SD) below the mean, even about 25 to 30 percent of patients has a BMD more than 2 SD below the mean (1, 7), whereas in pediatric patients with galactosemia the average is between 0 and -1 SD (5, 6). Fracture rates in adult patients with classic galactosemia have been reported between 31 and 63 percent (1, 7), which is slightly higher than in the general population in which rates between 21 and 53 percent have been reported for this age category (9). Only one study addresses the fracture rates in pediatric patients (7), and the reported prevalence of 18 percent is not higher than in the healthy pediatric population (10).

Although the pathophysiological mechanism is still not fully understood, several factors could negatively affect bone metabolism in this disease. Due to the galactose-restricted diet, patients are at risk of developing nutritional deficiencies. The primary ovarian insufficiency (POI) with low estradiol levels in females with galactosemia predisposes affected females to decreased bone mass. A disease specific intrinsic abnormality such as abnormal glycosylation of collagen or other glycoproteins involved in bone metabolism has also been suggested (4). Furthermore, the decreased IGF-I and IGFBP-3 concentrations that are found in galactosemia patients might be involved (11).

For classic galactosemia, optimization of exogenous factors influencing the bone mass is considered the most important intervention. The latest recommendations regarding follow-up and treatment of reduced bone mass in this disease date from 2007 (12). Since then, insights regarding different aspects of bone health have increased and there is a need to update and expand the previous recommendations based on our experience and the current literature. This proposal shortly reviews the diagnostic procedure and recommendations for follow-up including DXA, dietary evaluation, biochemical markers, physical activity, and in females supplementation of estrogen.

DIAGNOSTIC PROCEDURES

Dietary evaluation and bone metabolism markers

A balanced diet sufficient in energy, protein and micronutrients is a criterion for achieving and maintaining a high bone mass. Recently, it has been shown that body weight and Body Mass Index (BMI) correlate with bone mass in patients with classic galactosemia, emphasizing the need for monitoring caloric intake in this group (1). Evaluation of food records or food frequency questionnaires, preferably by a nutritionist, can be used to determine a patient's general nutrition status and to assess the completeness of a patient's diet.

The galactose-restricted diet predisposes patients to nutritional deficiencies, including a calcium deficiency. Dietary calcium intake therefore needs to be monitored, as is also recommended for other metabolic diseases in which the bone health is often affected. The recommended daily dietary calcium intake, which varies slightly per country, can be used as reference (table 1).

Adequate vitamin D status, assessed by dietary and laboratory evaluation, is also required for optimal bone health. The preferred marker for vitamin D status is the total 25-hydroxyvitamin D (25(OH)D) serum concentration. Measurement of 1,25(OH)2D is not recommended, since these concentrations do not reflect vitamin D reserves and can even be normal or elevated in patients with vitamin D deficiency due to secondary hyperparathyroidism (13). It is advised to aim for a total 25(OH)D serum concentration higher than 30 ng/mL (75 nmol/L) in patients at risk of developing osteoporosis and this requires a vitamin D intake of at least 400-1000 IU per day (13) (Table 1).

Table 1: Recommended dietary allowances of calcium (Institute of Medicine 2011) and vitamin D (Holick et al 2011) for different age groups in case of risk for low bone mass.

Age	Calcium	Vitamin D
<1 year	200-260 *	400-1000 IU/day
1-3 years	700 mg/day	600-1000 IU/day
4-8 years	1000 mg/day	600-1000 IU/day
9-13 years	1300 mg/day	600-1000 IU/day
14-18 years	1300 mg/day	600-1000 IU/day
19-70 years	1000 (M) or 1200 (F) mg/day	1500-2000 IU/day
>70 years	1200 mg/day	1500-2000 IU/day

* Reflects adequate intake value, since a recommended dietary allowance value has not been established for this group
M = value for males; F = value for females.

The importance of vitamin K in acquiring adequate bone mass remains controversial. Several studies, including one study in patients with classic galactosemia, have reported

that supplementation positively affects bone mass when combined with calcium and vitamin D. Panis et al (2006) showed that supplementation of vitamin K increased the concentration of carboxylated osteocalcin, which is required for the incorporation of calcium and hydroxyapatite in the bone (14). Braam et al (2003) found a slower bone loss in post-menopausal women receiving vitamin K in combination with calcium and vitamin D (15). However, there is also evidence that vitamin K does not increase bone mass (16).

Other nutrients that might be important for bone metabolism include zinc, phosphorus and magnesium. There have been concerns that soy formula might have a negative effect on bone metabolism and growth due to its high phytate content, which causes reduced absorption of zinc. However, in a recent meta-analysis, zinc serum concentrations were found to be similar in children who were fed soy infant formula during infancy compared to children fed human milk or cows' milk-based formula during infancy (17). Therefore, soy feeding related zinc deficiency is unlikely and measuring zinc concentrations is not recommended. Deficiencies of phosphorus and magnesium are unlikely in a balanced diet, and therefore routine monitoring is not advised for patients at risk of developing low bone mass. However, serum phosphate and serum magnesium require special attention when dietary assessment indicates an insufficient diet. Furthermore, there is some evidence that a high phosphorus intake, resulting from food additives, combined with a low or normal calcium intake may have adverse effects on bone metabolism due to a disturbed ratio of calcium-to-phosphorus intake (18). However, further research in this field is required to define adequate phosphate concentrations.

Other biochemical markers for bone metabolism, including carboxylated and uncarboxylated osteocalcin (cOC, ucOC), bone specific alkaline phosphatase (BAP), amino terminal telopeptide (NTX) and carboxy terminal telopeptide (CTX) are at this point only recommended in research settings.

Estrogen supplementation

In females, hypergonadotropic hypogonadism needs to be assessed by measuring FSH, LH and beta-estradiol concentrations. Estrogen is a powerful inhibitor of bone resorption via the alpha and beta estrogen receptors on osteoblasts and osteoclasts, and low levels negatively influence bone metabolism. Evaluation of these hormones and referral to a pediatric endocrinologist should start from the age of 10-12 years (19).

Physical activity evaluation

Sufficient physical activity is required to achieve optimal bone mass. The World Health Organization (WHO) recommends 60 minutes of moderate- to vigorous-intensity physical activity per day for children and 150 minutes per week for adults. However, this is

often not achieved. Furthermore, patients with classic galactosemia might be less physically active due to motor dysfunction (20) and less participation in social activities (21). Regular evaluation of physical activity in patients with this disease is therefore important and a standardized questionnaire, for instance the Physical Activity Questionnaire for Children and Adolescents (PAQ-C/A), can be used for this (22).

Assessment of spinal deformities

Scoliosis and hyperkyphosis are seen in 1-5 percent of all children in the general population and seem to be partly related to reduced bone mass and neurological abnormalities (23). Scoliosis is defined as a lateral curvature of the spine that is more than 10 degrees on a conventional X-ray in standing position. A hyperkyphosis is defined as a kyphosis of more than 45 degrees. We observed a prevalence of 29 percent of these spinal deformities in a cohort of patients with classic galactosemia (n=24, 14 females and 10 males, age range 13-48 years with a mean of 22 years), which is surprisingly higher than reported in the general population (unpublished results). A relationship between galactosemia and deformities of the spine has not been reported. It is questionable whether the observation in our cohort represents a real phenotypical characteristic or whether it is a coincidental finding. Yet, we advise to perform physical examination of the spine.

Dual energy X-ray absorptiometry

The method of choice to evaluate the bone mass is with dual energy X-ray absorptiometry (DXA) scans, since this is a safe and relatively easy method with low exposure to radiation and low costs. Recently, the 2013 recommendations of the International Society for Clinical Densitometry have been published (24, 25). For postmenopausal women and men aged 50 years or older DXA scans of the lumbar spine, femoral neck and total hip are advised and T-scores are preferred to express the decrease in BMD. However, in premenopausal women and men younger than 50 years of age Z-scores taking into account age, gender and ethnicity are preferred. Also for children Z-scores are preferred and the results should be adjusted in children with short stature or growth delay. Measurements of the BMD of the lumbar spine and the total body less head (TBLH) are recommended in children, since they yield the most reliable results. DXA scans of the femur have been proven to be unreliable in children and total body scans with head might give false positive outcomes due to the relative large size of the head during childhood.

In postmenopausal women and men aged 50 years or older, a BMD T-score ≤ -1.0 standard deviation (SD) is diagnostic of low bone mass (formerly called osteopenia), while a T-score ≤ -2.5 SD is diagnostic of osteoporosis. A BMD Z-score ≤ -2.0 SD in premenopausal women or males younger than 50 years of age is defined as a BMD below the expected range for age, whereas a Z-score > -2.0 SD represents a BMD within

the expected range for age (25). In children the diagnosis of osteoporosis requires either the finding of one or more vertebral compression fractures or the presence of both a BMD Z-score ≤ -2.0 SD and a clinically significant fracture history, meaning two or more long bone fractures by age 10 years, or three or more long bone fractures at any age up to 19 years. A Z-score < -2.0 SD without a clinically significant fracture is classified as a low BMD (24).

FOLLOW-UP AND THERAPEUTIC INTERVENTIONS

Dietary evaluation and bone metabolism markere

Batey et al (2013) found that most adults with galactosemia do not routinely visit a nutritionist, which might have implications for their bone health (1). Since a balanced diet is important for maximizing bone accrual during adolescence as well as for maintaining bone mass during adulthood, regular dietary evaluation throughout life is desirable. We recommend yearly dietary assessment, preferably by a nutritionist, with special focus on energy, protein, and micronutrient requirements. In case a patient's diet is suboptimal, enhanced nutritional counseling is essential to optimize a patient's general diet taking into account limitations deriving from galactose-restriction. Special focus on vitamin D and calcium intake is suggested, including yearly evaluation of total 25(OH)D serum concentrations. We recommend supplementation of calcium and vitamin D when intake is lower than the recommended daily intake and does not normalize despite dietary optimization. When vitamin D supplementation has been initiated, more frequent monitoring of 25(OH)D serum concentrations might be helpful in determining the optimal dosage. Supplementation of vitamin K might be beneficial when combined with an adequate intake of calcium and vitamin D, but currently there is not enough evidence to recommend the routine use of vitamin K supplements.

Estrogen supplementation:

If evaluation of hormone status indicates hypergonadotropic hypogonadism, initiation of hormonal replacement therapy (HRT) needs to be considered. The decision to start supplementation during adolescence will have to take into consideration growth, psychological factors and bone measurements. Duration of HRT throughout adult life will depend on an individual assessment of the possible risks and benefits.

Physical activity evaluation

We advise physicians to assess a patient's physical activity and to encourage exercise when evaluation suggests that the patient does not meet the recommendations. Im-

plementation of a regular routine of weight bearing exercise is recommended, taking into consideration possible physical limitations.

Assessment of spinal deformities

For now, we recommend physicians to evaluate for possible spinal deformities during routine physical examination. In case of clinical suspicion, an X-ray of the entire spine with a Cobb angle is recommended and subsequent referral to an orthopedist for extensive evaluation may be considered (26).

Dual energy x-ray absorptiometry

It is advised to assess BMD from an early age to ensure that optimal bone mass is reached in early adolescence. We recommend the first DXA to be performed at the age of 4 years, or as soon as the child is able to lie still, since a decreased bone mass can already be present at this early age. In children the minimum time interval between subsequent DXA scans is 6-12 months (ISCD 2013). We suggest yearly DXA scanning when BMD is ≤ -2.0 SD and one DXA scan per two years when BMD is higher. In adults it is advised to repeat a DXA scan one year after initiation or change of therapy (25). The interval can be increasingly elongated once the BMD is stable.

Furthermore, we recommend regular assessment of fracture history in order to determine whether a pediatric patient meets osteoporosis criteria and to evaluate the clinical relevance of a patient's reduced bone mass. In patients with frequent clinically relevant fractures additional pharmacological therapy needs to be considered.

CONCLUSION

Our recommendations regarding assessment and follow-up of decreased bone mass in patients with classic galactosemia (Figure 1):

- Dietary evaluation to assess adequacy of a patient's diet.
- Optimization of calcium intake, either improvement of dietary intake (preferably) or supplements.
- Monitoring of vitamin D status by dietary assessment and laboratory measurement of total 25(OH)D concentrations. If total 25(OH)D serum concentrations are < 30 ng/mL (< 75 nmol/L) intake should be improved through diet or supplements.
- Hormonal status evaluation in females, in case of hypergonadotropic hypogonadism HRT should be considered. Initiation and duration of treatment should take into consideration potential harms and benefits.
- Assessment of physical activity using standardized questionnaires. In case the WHO recommendations are not met, exercise needs to be encouraged.

- Evaluate for spinal deformities, since these might be more prevalent in this patient group, and fracture rate estimation.
- DXA as the tool to monitor BMD. DXA scans of the lumbar spine and TBLH are advised in children, whereas for adults DXA scans of the lumbar spine, femoral neck and total hip are preferred.

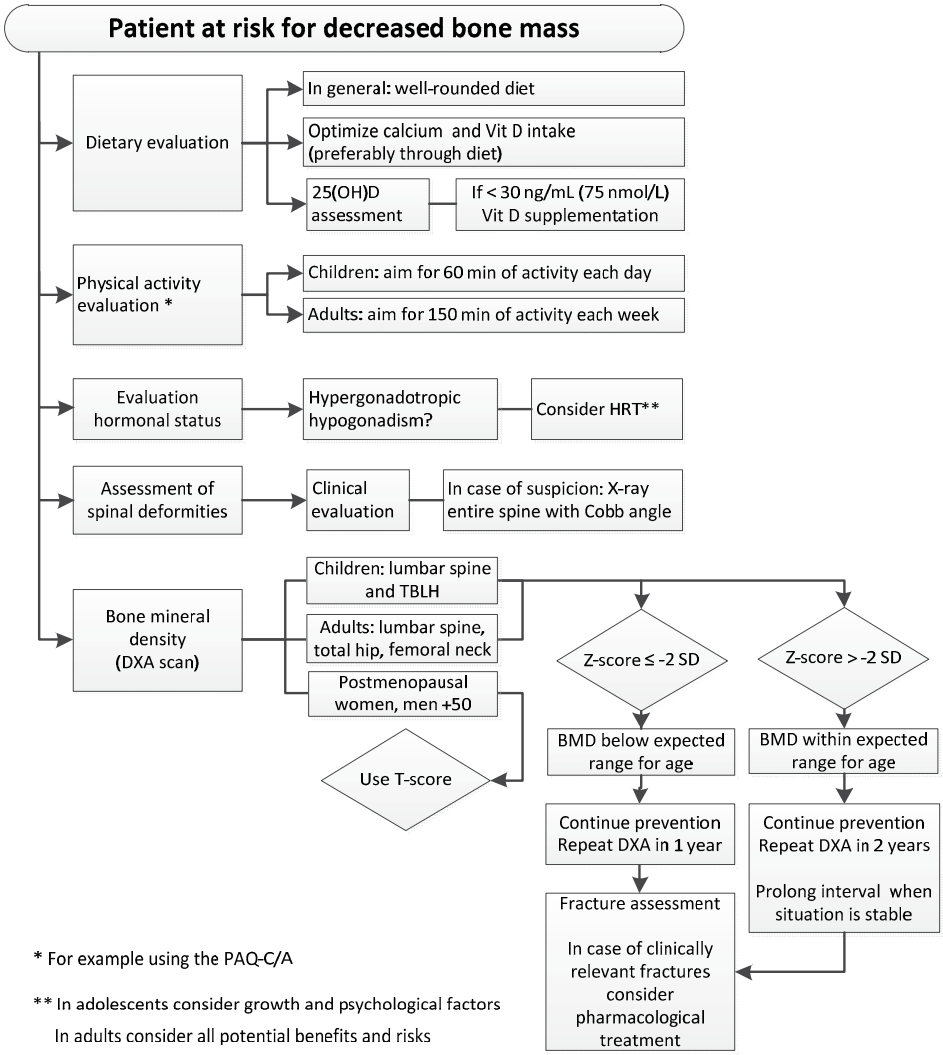

Figure 1: Flow chart for the follow-up of bone health in patients with classic galactosemia.

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Chapter 8

General discussion

The work presented in this dissertation aims to further investigate the chronic complications of classic galactosemia. The research builds up on previous work presented in other theses brought forth by the Galactosemia Research Group Maastricht. Objectives included the development of a new disease model, as well as improved understanding and patient care with regard to fertility, brain and bone impairments in this disorder.

DEVELOPMENT OF A ZEBRAFISH MODEL

In order to move classic galactosemia research forward, there was a high need for a novel disease model. The existing models, the *Drosophila melanogaster* model (1) and the mouse model (2), showed their importance for disease pathogenesis studies (3-8), yet do not meet the features demanded to fulfill two key investigations for the field: 1) organ studies throughout development in order to answer the long-standing open question if, and to what extent, prenatal organ damage occurs; 2) rapid and efficient testing of pharmacological compounds in order to establish new therapeutic approaches. The external development of the zebrafish, the availability of transgenic reporter lines and the accessibility to sophisticated imaging techniques make it an outstanding tool for organ studies from embryonic stage to adulthood. Furthermore, zebrafish models are amenable to high-throughput screening due to the large numbers of offspring, and are therefore increasingly used in pharmacological studies (9).

In chapter 2 we describe the generation of a *galt* knockout zebrafish model and demonstrate that our transgenic line recapitulates the human phenotype. At a biochemical level, an essentially complete lack of detectable galt enzyme activity and, upon exposure to exogenous galactose, accumulation of high concentrations of Gal-1-P were confirmed. The *galt* knockout fish develop reduced physical activity and a premature decline of egg production, which resembles the neurological sequelae and primary ovarian insufficiency found in humans with classic galactosemia.

Future investigations will encompass in-depth evaluation of damage to gonads and brain. Transgenic zebrafish lines that carry tissue-specific promoters driving green fluorescent protein (GFP) expression provide rapid, real-time *in vivo* developmental systems for analyzing tissue and organ development (9). Accordingly, to study gonads and brain, we crossed our *galt* knock-out transgenic zebrafish line to two reporter lines: a reporter line with fluorescent primordial germ cells (PGCs) (*vasa*:GFP) (10), and a reporter line with fluorescent myelin (*mbp*:GFP) (11). By generating a *galt* knockout line with labeled PGCs and a *galt* knock-out line with fluorescent myelin, we established two models to study the damage to gonads and brain from embryonic stage to adulthood. Future studies in these models (by Dr. Ana I. Coelho, Marie Curie postdoctoral fellow) will provide important information on when and to what extent damage to these target organs occurs.

Future investigations also encompass the exploration of glycosylation patterns and the implications of aberrant glycosylation for molecular pathways and organ function-

ing. Additionally, in-depth microscopic evaluation of the eye lens of *galt* knockout zebrafish is considered in order to evaluate the potential development of cataracts upon exposure to exogenous galactose (12).

Furthermore, a p.Q188R *galt* knock-in zebrafish will be developed to support the establishment of alternative therapeutic approaches such as pharmacological chaperones. Previous studies have demonstrated that enzymatic impairment of the most frequent GALT variants, including p.Q188R which affects about 60% of patients at a worldwide scale (13), results from altered protein folding, followed by aggregation and rapid destruction of the misfolded GALT protein (14-16). Pharmacological chaperones - molecules that have the ability to stabilize (misfolded) proteins (17-19) - have therefore been suggested as potential therapeutic agents for this disorder (14, 15). By changing protein conformation, the stability and activity of the protein is increased. Since individuals with a GALT activity of >10% are generally not considered patients (20), only a slight elevation of residual enzyme activity might be sufficient to prevent chronic impairments. As a result of the high prevalence of p.Q188R within the galactosemia population, many patients could potentially benefit from a chaperone treatment. The p.Q188R *galt* knock-in zebrafish model is designed to evaluate the effect of pharmacological chaperones using a high-throughput screening approach. One of the compounds of interest is arginine, an amino acid with high therapeutic potential as a pharmacological chaperone and outstanding *in vitro* results (21).

OVARIES

The second aim of this dissertation was to further investigate the clinical implications of primary ovarian insufficiency (POI), which is present in nearly all females with classic galactosemia from an early age on and entails the struggle of having reduced pregnancy chance. Though POI reflects a continuum of impaired ovarian function, which is varying, unpredictable and does not exclude pregnancy (22, 23), fertility counseling of women with classic galactosemia and POI has remained of discouraging nature throughout the past years. In chapter 3 we present the first international, large-scaled epidemiological study on fertility in these patients. Our findings suggest that pregnancy rate is higher than expected, with a prevalence of 42.9% in our cohort, even though many women did not try to conceive due to negative counseling. If women with classic galactosemia and POI try to conceive for a longer period of time (>2 years) as compared to couples in general, it is estimated that >60% conceive within 2.5 years. These data fortify the results of earlier work from our group (24, 25) and underline the paradigm that these women have a significant chance of spontaneous conception. Accurate fertility counseling is thus important to encourage pregnancy attempts in those with an active child-wish and to prevent unplanned pregnancies in those who have no intention of ever having children.

We evaluated several characteristics that could potentially affect an individual's chance of spontaneous conception, including a history of a spontaneous menarche and the p.Q188R/p.Q188R mutation. However, our data did not confirm a statistically significant effect of any of these features. Furthermore, anti-Müllerian hormone (AMH) concentrations and ovarian imaging results (MRI, ultrasound) do not accurately predict fertility outcome in this population (26, 27). Taken together, adequate markers of future pregnancy chance are lacking, which should be taken into account in reproduction counseling.

POI and the consequent chance of reduced fertility are considered a major burden by both patients and their parents (28). As a result, many questions exist regarding the option to preserve fertility. Consensus on the application of fertility preservation techniques in this population was lacking, and therefore we felt there was a need to develop recommendations, elaborated with the support of an international multidisciplinary expert team (chapter 4). We proposed that the uncertainty surrounding the success of fertility preservation in this group and the significant chance of spontaneous pregnancy warrant counseling towards conservative application of these techniques. In case fertility preservation is deeply wished for, cryopreservation of ovarian tissue in young prepubertal girls in a research setting currently could be considered. In order to improve knowledge on long-term outcome and establish revised recommendations in the future, reports of longitudinal follow-up of patients after ovarian tissue cryopreservation in a research setting is important.

As briefly discussed in chapter 4, another potential approach for galactosemic women to preserve their fertility is oocyte donation, often by a family member or acquaintance. Though adult patients can seek for donors when pregnancy is desired, there is a tendency of intrafamilial egg donation at a very early stage, i.e. a mother donating oocytes to her (newborn) girl with classic galactosemia. Though from a medical perspective this early intervention is needed to secure high quality of the donated oocytes, the procedure of a mother donating to her young daughter can be considered concerning. Ethical dilemmas that may arise include psychosocial pressure on the mother (to donate oocytes) and the recipient (to use the donated eggs), over-attachment of the donor to the offspring, and role confusion for all persons involved, including the resulting offspring (29, 30). In addition, galactosemia-specific issues require consideration, including the daughter's unpredictable chance of developing cognitive impairments and psychosocial difficulties later in life (31-40). Thorough evaluation of the potential benefits and risks by a multidisciplinary group is currently ongoing.

BRAIN

The third objective of this thesis was to further improve our understanding of the pallet of brain impairments encountered by patients with classic galactosemia. Chapter 5 is

dedicated to a study within the field of cognitive neuroscience that builds up on previous work by our group (41). These earlier investigations used innovative magnetic resonance imaging (MRI) techniques to demonstrate structural abnormalities of white and grey matter in this disorder. Also, alterations in functional brain networks involved in speech and language production were revealed during a language task. In addition to task-based functional MRI (fMRI) studies, resting-state fMRI investigations, in which neuronal connectivity in absence of a specific stimulus or task is assessed, are increasingly conducted within multiple fields of neuroscience to study the organization of core processing systems of the brain. These investigations allow exploration of the overall functional organization of brain networks, which had not been studied before in classic galactosemia. Therefore, we conducted a resting-state fMRI study in 13 adolescent patients and 13 healthy age- and gender-matched controls. A seed-based correlation analysis (SCA) was used to evaluate intrinsic functional brain networks during rest. Results showed robust group differences in resting-state functional networks involved in sensory-motor integration, (visuo)spatial orientation and capacities, motor (speech) planning, language production and working memory. Most importantly, across several seed analyses, altered functional connectivity to the lingual gyrus, the precentral gyrus, the insula and the cuneus was observed in patients, suggesting these brain regions might be of special relevance. In addition, these differences in network connectivity correlated with clinical test results in patients, supporting a relation between functional abnormalities and the clinical phenotype. In the future, more thorough investigations of these specific brain regions are needed to evaluate their potential role in pathogenesis.

In the current study, eight brain regions were *a priori* selected as seeds for a SCA, based on previous studies indicating altered structure, function and/or metabolic activity of these regions (42-44). Future fMRI studies might consider an independent component analysis (ICA) approach, which is data-driven and examines the general pattern of unique functional connectivity across the brain, without selection of a specific region of interest/seed (45-47). As a result, this approach might yield a more extensive evaluation of resting-state networks. Additionally, future studies are required to thoroughly investigate functional connectivity of the cerebellum at rest, since this brain structure was not entirely included in the functional coverage of the current study. Though functional connectivity of the cerebellum was not particularly affected in our study, cerebellar involvement is often suggested to be involved in the pathogenesis of neurological sequelae, motor problems and potentially language and speech disorders (48, 49).

Our findings contribute to the characterization of the functional brain impairments in this enigmatic disease. However, in order to provide more detailed hypotheses on the pathogenesis of these deficits, investigations of longitudinal data and a systematic comparison of results of structural and functional brain imaging studies are needed. Longitudinal MRI data could provide information on the general disease process and progression over time. As a result, these data may give insight in whether future performance and disease outcome at an individual level can be predicted, which is espe-

cially important for the development of tailored treatment approaches. By combining data on white and grey matter structure with functional network assessments, it might become more clear whether the fMRI alterations represent specific targeted damage, or whether brain regions/networks with aberrant functional connectivity are equally affected as other regions but show a more prominent functional impairment. Alternatively, these differences in functional connectivity might reflect compensation mechanisms to cope with cognitive difficulties.

BONE

With this dissertation we also aimed to gain more insight in the extent and clinical implications of impaired bone health in this disease. The first report of an association between classic galactosemia and decreased bone mass dates back to 1993 (50), and the Galactosemia Research Group Maastricht extensively studied this long-term complication in the past (51). However, its extent and relevance for patient care remained undefined due to limited sample sizes of previous investigations. Therefore, we conducted a systematic review and meta-analysis on bone mineral density (BMD) in adults and children with classic galactosemia, which is presented in chapter 6. Results indicate that bone health in general is mildly affected, and that an estimated proportion of 10-25% of patients could be at risk for a low bone mass (BMD Z-score ≤-2). As a result, it is important that treating physicians are aware that patients are at risk for having or developing low bone mass, warranting appropriate screening and treatment.

Thus far, the pathogenesis of bone mass reduction in this disorder remains poorly understood. Since nutritional deficiencies and decreased estrogen concentrations alone do not seem to fully explain the low bone mass in this population (50, 52, 53), an intrinsic bone defect due to aberrant glycosylation has been suggested (50, 54-56). Evaluation of glycosylation patterns of proteins and hormones involved in bone metabolism could shed new light on the pathogenesis, thereby providing relevant information for bone health management.

As a result of evolved experience with classic galactosemia and improved knowledge on bone mass in general, we felt the 2007 recommendations on bone health monitoring in this group required an update (57). A revised strategy for diagnosis, treatment and follow-up of reduced bone mass in this population is outlined in chapter 7. Frequent evaluation of nutritional status, vitamin D levels, calcium intake, estrogen concentrations and physical activity, as well as regular monitoring of BMD using dual-energy X-ray absorptiometry (DXA) scans, were suggested. We advised yearly DXA scanning in case of low bone mass and one scan per two years in case of adequate bone mass, with elongation of the intervals once BMD is considered stable. Evolved insights on the estimated proportion of patients at risk for low bone mass suggest that the DXA monitoring frequency could be reduced, particularly in those patients with accurate bone mass. Fu-

ture scientific investigations and improved clinical experience will have to define an optimal DXA monitoring rate.

In chapter 7 we suggest a possible association between classic galactosemia and spinal deformities, such as scoliosis and hyperkyphosis. We observed that 29% of patients from our cohort suffered from spinal deformities, whereas a prevalence of 1-5 % is reported in the general population (58). Development of these deformities has been associated with, among others, impaired strength of connective tissue, (paraspinal) muscle abnormalities, reduced bone mass of the lumbar spine and epigenetic factors (59-61), which could have a role in the increased prevalence observed in our cohort. Systematic evaluation of the prevalence of spine deformities in a large cohort, and comparison to a matched control group or the general population, is needed to explore this potential deficit. It is expected that the patient registry of the Galactosemia Network (GalNet), an international network of galactosemia treatment centers from 18 European countries, the United States of America (USA) and Australia (www.galactosemianetwork.org), will provide large-scaled, cross-sectional insights in this matter, as well as in many other aspects of the clinical phenotype of patients with this metabolic disorder.

CONCLUSIONS

The studies presented in this dissertation provide new perspectives on the chronic impairments of ovaries, brain and bone in classic galactosemia through a combination of basic and clinical research. We successfully developed a galt knockout zebrafish line, which recapitulates the human phenotype. With this model we generated an outstanding tool for studies of organ damage throughout development and high-throughput screening of pharmacological compounds. Furthermore, we demonstrated that pregnancy rate in adult women with classic galactosemia and POI is higher than expected, which has significant implications for counseling of these patients and their families regarding reproduction and fertility preservation. We also evaluated the application of fertility preservation techniques in this patient group and recommended conservative application, based on the significant chance of spontaneous conception and the unknown success rates of preservation methods. Additionally, we further characterized the pallet of brain impairments encountered by patients with classic galactosemia through a resting-state fMRI study. We demonstrated robust differences as compared to controls that are in line with the neurocognitive profile of patients, thereby providing directions for future, in-depth investigations. Previous small studies suggested an association between classic galactosemia and low bone mass, but evaluation of the extent and clinical implications in a large cohort was lacking. Findings from our systematic review and meta-analysis fortify the earlier conjecture of a mildly decreased bone mass in this population, which warrants clinical awareness. In order to prevent low bone mass in these patients, recommendations on bone health monitoring and optimization were proposed.

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List of abbreviations

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
ACC	anterior cingulate cortex
ADHD	attention deficit hyperactivity disorder
ANOVA	analysis of variance
BMC	bone mineral content
BMD	bone mineral density
BOLD	blood oxygen level dependent
BTM	bone turnover marker
CG	classic galactosemia
CI	confidence interval
cOC	carboxylated osteocalcin
CSF	cerebral spinal fluid
DXA	dual energy X-ray absorptiometry
EPI	echo-planar imaging
ERP	event related potential
Fc	functional connectivity
FDG-PET	[18F]fluorodeoxyglucose positron emission tomography
(f)MRI	(functional) magnetic resonance imaging
FSH	follicle stimulating hormone
Gal-1-P	galactose-1-phosphate
GALE	UDP-galactose 4'-epimerase
GALK	galactokinase
GALT	galactose-1-phosphate uridylyltransferase
GFP	green fluorescent protein
Glc-1-P	glucose-1-phosphate
HRT	hormone replacement therapy
IFG	inferior frontal gyrus
IPD	individual patient data
IPL	inferior parietal lobule
ISCD	International Society for Clinical Densitometry
ISCED	International Standard Classification of Education
LH	luteinizing hormone
MCC	mid cingulate cortex
mPFC	medial prefrontal cortex
MeFG	medial frontal gyrus
MFG	middle frontal gyrus

MTG	middle temporal gyrus
OR	odds ratio
PCC	posterior cingulate cortex
PGC	primordial germ cell
POI	primary ovarian insufficiency
PoG	postcentral gyrus
(pre-)SMA	(pre-)supplementary motor area
PrG	precentral gyrus
(q)PCR	(quantitative real-time) polymerase chain reaction
RCT	randomized controlled trial
RFX	random-effects analysis
ROI	region of interest
RT	reaction time
SCA	seed-based correlation analysis
SD	standard deviation
SFG	superior frontal gyrus
SPL	superior parietal lobule
STG	superior temporal gyrus
TALEN	transcription activator-like effector nuclease
TBLH	total body less head
TE	echo time
TR	repetition time
ucOC	under-carboxylated osteocalcin
UDP-gal	UDP-galactose
VOI	volume of interest
WM	white matter

Summary

Classic galactosemia is a genetic disorder of galactose metabolism due to profound deficiency of the enzyme galactose-1-phosphate uridylyltransferase (GALT). The current standard of treatment - a galactose-restricted diet - fails to prevent the development of burdensome impairments of fertility, brain and bone health. In chapter 1 we further elaborate on this disorder and its complications, and introduce the objectives of this thesis. Our objective was to develop an animal model that allows organ studies throughout development and pharmacologic compound testing. Furthermore, the work presented in this dissertation aimed to expand clinical insights on fertility, brain and bone health complications, to improve patient care and counseling.

In order to move classic galactosemia research forward, there was a high need for a novel disease model, since the existing models are less amenable to organ studies throughout development and high-throughput screening of pharmacologic compounds. Chapter 2 describes the generation of a *galt* knockout zebrafish model and its phenotypical characterization. Using a TALEN approach, knockout of the *galt* gene was successfully established. Analogous to humans, biochemical assays confirmed essentially undetectable galt enzyme activity and, upon exposure to exogenous galactose, accumulation of galactose-1-phosphate in knockout fish. Moreover, *galt* knockouts exhibited reduced motor activity and a premature decline of egg production, resembling the human phenotype of neurological sequelae and ovarian damage. In conclusion, this model mimics the human phenotype at biochemical and clinical levels and will contribute to improved understanding and management of this disorder.

The next two chapters of this dissertation focus on fertility impairments in female patients. Primary ovarian insufficiency (POI) comprises a continuum of impaired ovarian function and represents the most frequent long-term complication of classic galactosemia. For years, chance of spontaneous conception was assumed to be marginal for these women, yet spontaneous pregnancies have been reported regularly in this group. In chapter 3 we present the results of an international, epidemiological study on fertility in a cohort of 85 women with classic galactosemia and POI. Many women did not try to conceive, because they were told they could not have children. In those women that attempted to conceive, pregnancy rate was higher than expected (9/21 = 42.9%). This shifting paradigm carries significant implications for reproduction counseling and the application of fertility preservation techniques in this group.

Physicians are often asked about possible options to preserve fertility in female patients. However, due to the lack of guidelines on fertility preservation in this population, there is a great diversity of approaches around the world. Chapter 4 addresses recommendations on fertility preservation in girls and women with classic galactosemia, developed with the support of an international multidisciplinary expert team. We propose that the uncertainty surrounding success rates of fertility preservation and the likely significant chance of spontaneous pregnancy warrant counseling towards conservative application of these techniques. If preservation of fertility is truly wished for, cryopreservation of ovarian tissue in young prepubertal girls in a research setting currently

seems the best option, since cryopreservation of oocytes at a later age is likely unsuccessful.

The brain represents the other main target of organ damage in classic galactosemia. Despite decades of research, only little is known about the neural correlates of the pallet of cognitive impairments encountered by these patients. Chapter 5 encompasses an explorative functional magnetic resonance imaging (fMRI) study on resting-state neural networks. With this study, we are the first to investigate functional connectivity during rest in this population, using a seed-based approach. Resting-state networks were characterized in 13 adolescent patients and 13 healthy controls, and results point towards several substantial group differences. Alterations were observed in resting-state functional networks involved in sensory-motor integration, (visuo)spatial capacities, motor (speech) planning, language production, and working memory, which is in line with the neurocognitive profile of patients. Most importantly, across several analyses, repeatedly altered connectivity of some brain regions was observed (lingual gyrus, precentral gyrus, insula and cuneus), suggesting these brain regions might be of special relevance. These findings provide directions for future in-depth studies, aiming to further elucidate the role of these key brain regions in disease pathogenesis. Also, our results shed light on new potential targets for clinical interventions, including (visuo)spatial capacities, which require further exploration in clinical studies.

Additionally, previous investigations indicate an association between classic galactosemia and decreased bone mass. However, these studies were of limited sample size and results varied, leaving the extent of bone health impairment and its relevance for patient care undefined. We conducted a systematic review and meta-analysis on bone mineral density (BMD) in adults and children with classic galactosemia to evaluate bone health in this population and the need of monitoring and treatment (chapter 6). We demonstrate that bone health in general is mildly affected (mean BMD Z-score = -0.7) and that an estimated proportion of 10-25% of patients with classic galactosemia could be at risk for a low bone mass (BMD Z-score ≤-2). Therefore, follow-up and treatment are warranted.

A revised strategy for diagnosis, treatment and follow-up of reduced bone mass in this population is presented in chapter 7. We recommend regular evaluation of nutritional status and diet, physical activity, and, in females, ovarian function. Serial dual-energy X-ray absorptiometry (DXA) scans are advised to accurately monitor BMD.

Finally, in chapter 8, results are discussed and directions for future research are suggested.

Samenvatting

Klassieke galactosemie is een aangeboren aandoening van de galactosestofwisseling, veroorzaakt door ernstige deficiëntie van het enzym galactose-1-fosfaat uridylyltransferase (GALT). De huidige behandelstandaard – een galactose-beperkt dieet – kan ernstige beperkingen van vruchtbaarheid, brein en botgezondheid niet voorkomen. In hoofdstuk 1 gaan we verder in op deze aandoening en haar complicaties en introduceren we de doeleinden van dit proefschrift. Doelstelling was een diermodel te ontwikkelen dat kan voorzien in orgaanstudies gedurende verschillende levensfasen en het testen van farmacologische stoffen. Bovendien beoogde dit proefschrift de klinische inzichten in de complicaties van eierstokken, brein en botten te vergroten om de patiëntenzorg en counseling te verbeteren.

Om het onderzoek naar klassieke galactosemie een impuls te kunnen geven, was er grote behoefte aan een nieuw diermodel. De reeds beschikbare diermodellen (fruitvlieg en muis) zijn minder geschikt voor het bestuderen van organen gedurende verschillende levensfasen en voor het snel en efficiënt screenen van farmacologische stoffen. Daarom besloten wij een zebravismodel voor klassieke galactosemie te creëren. Hoofdstuk 2 beschrijft de ontwikkeling van een *galt* knockout zebravismodel en de typering van het fenotype. Succesvolle uitschakeling van het *galt* gen (knockout) werd bereikt middels een TALEN methode. Net als mensen met klassieke galactosemie hadden knock-out vissen een niet-detecteerbare galt enzymactiviteit. Tevens vond na blootstelling aan galactose forse stapeling van galactose-1-fosfaat plaats in het lichaam. Bovendien lieten *galt* knockout vissen een verminderde lichamelijke activiteit en een vroegtijdige afname van de eitjesproductie zien, overeenkomstig aan het beeld van neurologische afwijkingen en eierstokschade in mensen met klassieke galactosemie. Concluderend bootst dit model het menselijke fenotype na op zowel biochemische als klinische niveaus. Toekomstige studies in dit zebravismodel zullen onze kennis over de ziekte vergroten en zullen bijdragen aan een betere behandeling van de aandoening.

De volgende twee hoofdstukken van dit proefschrift richten zich op de vruchtbaarheidsproblemen in vrouwelijke patiënten. Primaire ovariuminsufficiëntie (POI) omvat een breed scala aan symptomen van verminderde eierstokfunctie en is de meest frequente lange termijn complicatie van klassieke galactosemie. Jarenlang werd gedacht dat de kans op spontane zwangerschap voor deze vrouwen nihil was. Echter, spontane zwangerschappen zijn wel degelijk regelmatig gerapporteerd in deze groep. In hoofdstuk 3 presenteren we de resultaten van een internationale, epidemiologische studie naar vruchtbaarheid in 85 vrouwen met klassieke galactosemie en POI. Veel van deze vrouwen hadden niet geprobeerd zwanger te worden, omdat hen was verteld dat ze geen kinderen konden krijgen. In de vrouwen die hadden gepoogd zwanger te worden, was het aantal zwangerschappen hoger dan verwacht (9/21 = 42,9%). Deze verschuivende opvatting over zwangerschapskans brengt aanzienlijke gevolgen met zich mee voor reproductiecounseling en de toepassing van fertiliteitspreservatie in deze groep.

Aan behandelend artsen wordt vaak gevraagd of er mogelijkheden bestaan om de vruchtbaarheid van vrouwelijke patiënten te waarborgen. Echter, door het gebrek aan richtlijnen over fertiliteitspreservatie in deze populatie bestaat er wereldwijd een grote diversiteit in aanpak. Hoofdstuk 4 beschrijft aanbevelingen voor fertiliteitspreservatie in meisjes en vrouwen met klassieke galactosemie, die tot stand zijn gekomen met de ondersteuning van een internationaal multidisciplinair expertteam. De onbekende slagingskansen van fertiliteitspreservatie en de aanzienlijke kans op spontane zwangerschap vragen om counseling richting een terughoudende toepassing van deze technieken. Als de wens om preservatie van fertiliteit groot blijft, lijkt op dit moment het invriezen van eierstokweefsel in jonge, pre-puberale meisjes, in de setting van een wetenschappelijk onderzoek, de beste optie. Het invriezen van eicellen op latere leeftijd is waarschijnlijk niet succesvol als gevolg van de op dat moment reeds vergevorderde eierstokschade.

Het andere hoofddoelwit van orgaanschade in klassieke galactosemie is het brein. Ondanks vele jaren van onderzoek is weinig bekend over de mechanismen onderliggend aan het scala aan cognitieve beperkingen die deze patiënten kunnen ontwikkelen. Hoofdstuk 5 omvat een exploratieve functionele MRI studie, waarbij voor de eerste keer de breinnetwerken in rust in deze populatie worden bestudeerd. Middels een seed-based benadering werden functionele netwerken in rust gekarakteriseerd in 13 jongvolwassen patiënten en 13 gezonde controles, waarbij enkele substantiële groepsverschillen werden gevonden. Veranderingen werden gezien in netwerken betrokken bij de integratie van het sensorische en motorische systeem, (visuo)spatiële functies, motorische (spraak)planning, taalproductie en werkgeheugen. Dit is in lijn met het neurocognitieve profiel van mensen met klassieke galactosemie. Enkele hersengebieden (gyrus lingualis, pre-centrale gyrus, insula en cuneus) toonden herhaaldelijk abnormale functionele verbindingen in patiënten, wat erop kan duiden dat deze gebieden van bijzondere relevantie zijn. De huidige bevindingen bieden aanknopingspunten voor toekomstige onderzoeken, die de rol van deze hersengebieden in de ontstaanswijze van de ziekte verder kunnen ontrafelen. Ook wijzen onze resultaten op mogelijke nieuwe doelwitten voor interventies, waaronder de (visuo)spatiële vaardigheden, die echter eerst verdere bestudering behoeven in klinische studies.

Daarnaast boden eerdere onderzoeken aanwijzingen voor een samenhang tussen klassieke galactosemie en verlaagde botmassa. Deze studies waren echter van beperkte grootte en de resultaten van de verschillende onderzoeken varieerden, waardoor de mate van botdichtheidsafname en de klinische relevantie nog altijd onduidelijk bleven. Wij voerden een systematische review en meta-analyse uit naar botdichtheid in volwassenen en kinderen met klassieke galactosemie om de botgezondheid in deze populatie te evalueren en de noodzaak tot monitoring en behandeling vast te stellen (hoofdstuk 6). Wij toonden aan dat de botgezondheid over het algemeen mild is aangetast en dat naar schatting 10-25% van de patiënten risico loopt op een te lage botmassa. Derhalve zijn monitoring en behandeling van botdichtheid in deze groep gerechtvaardigd.

Een herziende strategie voor diagnostiek, behandeling en follow-up van verminderde botmassa in deze populatie wordt gepresenteerd in hoofdstuk 7. Wij adviseren regelmatige evaluatie van voedingsstatus en dieet, lichamelijke activiteit en, in vrouwelijke patiënten, eierstokfunctie. Herhaaldelijke botscans worden aanbevolen om de botdichtheid accuraat te monitoren.

Ten slotte worden in hoofdstuk 8 alle resultaten bediscussieerd en worden suggesties voor toekomstige vervolgonderzoeken gegeven.

Valorization

The work presented in this dissertation aimed to further investigate the chronic impairments of classic galactosemia using a combined approach that encompasses both basic and clinical research. In this addendum the valorization potential of this thesis is presented.

Upon exposure to galactose in dairy milk, neonates with classic galactosemia develop a potentially lethal syndrome that resolves when galactose-restriction is initiated timely. Notwithstanding its irrefutable life-saving role in the newborn phase, the current standard of therapy – a lifelong galactose-restricted diet – is unsatisfactory, since it fails to prevent damage to ovaries, brain and bones. Importantly, even those patients who were never exposed to galactose develop long-term impairments (1, 2), suggesting a more sophisticated therapy is needed to overcome the complex disease pathogenesis. The phenotypic spectrum is diverse, ranging from patients who are severely affected and unable to live independently to patients who experience only mild symptoms. In general, the chronic debilitations negatively influence quality of life in the galactosemia population, and they form a major concern for the patients' parents as well (3). Furthermore, educational levels and employment rates are lower in patients, leading to impaired societal functioning (4). Due to the high costs of disease monitoring and treatment, the complications of classic galactosemia also form a burden for the healthcare system. As a result, the ultimate aim of galactosemia research worldwide is the development of new therapies that can prevent these long-term impairments and their negative impacts on patients and their families, society and the healthcare system.

Thus far, the timing of onset of organ damage in this disorder – more specifically the presence or absence of prenatal toxicity – remains to be elucidated. The existing disease models, the mouse and *Drosophila melanogaster* models (5, 6), showed their importance for disease pathogenesis studies (7-11), yet fail to meet the features demanded to evaluate whether damage has a prenatal and/or postnatal origin. Furthermore, these models are less amenable to testing pharmacologic compounds through a high-throughput screening approach. Therefore, the first objective of this dissertation was to develop a novel disease model for classic galactosemia. For this purpose, we developed a *galt* knockout zebrafish model. The model mimics the human phenotype at both biochemical and clinical levels, making it suitable to study the onset of organ damage and to develop new therapeutic strategies.

Throughout the process of this thesis, we developed the *galt* knockout zebrafish model and crossed our model to reporter lines. Future studies in this disease model will focus on the organ-specific onset of damage (gonads and brain) and time-specific extent of damage, in order to answer the long-standing open question if, and to what extent, prenatal organ damage occurs in this disorder. The many advantageous features of the zebrafish allow the application of innovative study designs and analysis techniques. Transgenic zebrafish lines that carry tissue-specific promoters driving green fluorescent protein (GFP) expression provide rapid, real-time *in vivo* developmental systems for analyzing tissue and organ development (12). Accordingly, to study damage

to gonads and brain, we crossed our *galt* knockout transgenic zebrafish line to two reporter lines: a reporter line with fluorescent primordial germ cells (PGCs) (*vasa*:GFP) (13), and a reporter line with fluorescent myelin (*mbp*:GFP) (14). By generating a *galt* knockout line with labeled PGCs and a *galt* knockout line with fluorescent myelin, we established two excellent models to study the damage to female gonads and brain from embryonic stage to adulthood.

Secondly, our zebrafish model is amenable to a high-throughput screening approach, enabling rapid and efficient testing of pharmacologic compounds of interest. As a result, the zebrafish is increasingly used as a disease model in pharmacologic studies, with experience rapidly evolving (12). Hitherto, several potential therapeutic agents have been proposed for classic galactosemia treatment, including galactokinase inhibitors and pharmacologic chaperones (15-18). Previous studies demonstrated that enzymatic impairment of the most important GALT variants results from altered protein folding, followed by aggregation and rapid destruction of the misfolded GALT protein (18-20). Pharmacologic chaperones - molecules that have the ability to stabilize (misfolded) proteins (21-23) - are therefore suggested as potential therapeutic agents for this disorder (18, 19). By changing protein conformation, the stability and activity of the protein is increased. Since individuals with a GALT activity of >10% are generally not considered patients (24), only a slight elevation of residual enzyme activity might be sufficient to prevent chronic impairments. One of the compounds of interest is arginine, an amino acid with high therapeutic potential as a pharmacologic chaperone and outstanding *in vitro* results (15). Future studies are planned to assess arginine's potential as a chaperone treatment for classic galactosemia in our zebrafish model. For this, a *galt* p.Q188R knock-in model will be developed.

Since the establishment of well-validated therapies is a long-lasting process, it is highly important that current patient care is optimized and that interventions are developed to maximize patient functioning despite impairments. Previous research showed that many differences exist with regard to diagnosis, treatment and follow-up of patients with classic galactosemia (25). The second aim of this thesis was to further investigate the extent and clinical implications of the damage to ovaries, brain and bones, thereby providing recommendations for improvement of patient care.

Ever since the report on a remarkably high prevalence of hypergonadotropic hypogonadism in women with classic galactosemia in 1979 (26), female patients were counseled for infertility from an early age on. Current findings from our international, epidemiological study on pregnancy chance in women with classic galactosemia and primary ovarian insufficiency indicate this counseling approach is incorrect. We showed that, despite ovarian damage, these women have a considerable chance of spontaneous conception. This shifting paradigm has significant implications for fertility counseling and the potential application of fertility preservation techniques. In the future, female patients and their families/parents should be counseled for reduced fertility rather than for infertility, which is the current practice.

Physicians are often asked about possible options to preserve fertility in female patients. However, due to the lack of guidelines on fertility preservation in this population, there is a great diversity of approaches around the world. Therefore, we evaluated the risks and benefits of fertility preservation in females with this disorder and made recommendations on how to address this important matter. With the support of a multidisciplinary expert team, we suggested a conservative approach towards fertility preservation through invasive techniques, given the significant chance of spontaneous conception. If preservation of an individual's fertility is truly wished for, ovarian tissue cryopreservation at an early age in a research setting seems the best option, due to the early depletion of the follicle pool.

In addition, we were the first to investigate functional connectivity during rest in this population, using functional magnetic resonance imaging. We evaluated resting-state functional brain networks in patients and matched controls. Our results point towards several substantial group differences, with repeatedly altered connectivity of some brain regions across analyses. Importantly, the affected networks are known to be involved in motor (speech) planning, visuospatial processing, working memory and language processing, which is in line with the neurocognitive profile of these patients. These findings can form the basis for future in-depth studies and the development of new interventions, targeting the affected cognitive functions (speech and language, working memory, sensory-motor integration, visuospatial capacities). Our data suggest patients might benefit from visuospatial training, which has not yet been implicated before, but clinical studies are needed to further explore this hypothesis.

Our meta-analysis on bone mineral density was the first study to provide adequate evidence for mild bone mass reduction in classic galactosemia. Previous studies were underpowered due to small sample sizes and results varied across studies (27-30), hampering solid conclusions on severity and clinical relevance of bone mass reduction in this disorder. Our findings warrant clinical awareness and monitoring of bone health, since 10-25% of patients are at risk of developing low bone mass. We also developed recommendations on diagnosis, treatment and follow-up of bone health in order to encourage consensual and accurate patient care worldwide. Implementation of these data on bone mass and bone health monitoring in future international guidelines is of importance.

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Curriculum vitae

Britt van Erven was born on February 14th 1992 in Geldrop, the Netherlands. She graduated *cum laude* from high school (gymnasium) at the Carolus Borromeus College in Helmond in 2009, after which she started the Bachelor program in Medicine at Maastricht University.

In her third year, she started as a student-assistant within the research group of Dr. Estela Rubio-Gozalbo on classic galactosemia. At the beginning of her Master program in Medicine (2012), during her internship at the Department of Pediatrics, section Metabolic Diseases, of the Maastricht UMC+, she started working as a PhD candidate under supervision of Dr. Estela Rubio-Gozalbo. She conducted her PhD research primarily in parallel to her Master program in Medicine. Two three-month-periods were scheduled off to work on research fulltime (2013 and 2014), which was financed by a grant from the Stichting Kindergeneeskunde, galactosemia reseach budget. After she graduated *cum laude* from Maastricht University in January 2016, she continued her work as a PhD candidate fulltime for a period of eight months to complete her research projects.

During her PhD trajectory, Britt received a grant from the Dutch/Belgian Inborn Error Association for her work in zebrafish in 2013. In 2015, she received a travel scholarship to visit the Symposium for the Society for the Study of Inborn Errors of Metabolism in Lyon. Furthermore, she received a travel grant from GROW School for Oncology and Developmental Biology, Maastricht University to visit the United States of America for her project on fertility in classic galactosemia (2016). Moreover, she was selected for the TULIPS PhD Curriculum 2016-2018, a two-year-program for PhD researchers with the potential and ambition to advance their research field as a clinician-scientist.

Currently, Britt is working as a resident at the Internal Medicine Department of the Máxima Medical Center in Veldhoven as part of her specialty training, coordinated by Maastricht UMC+. Together with Ben Jansen, she lives in Eindhoven.

List of publications

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